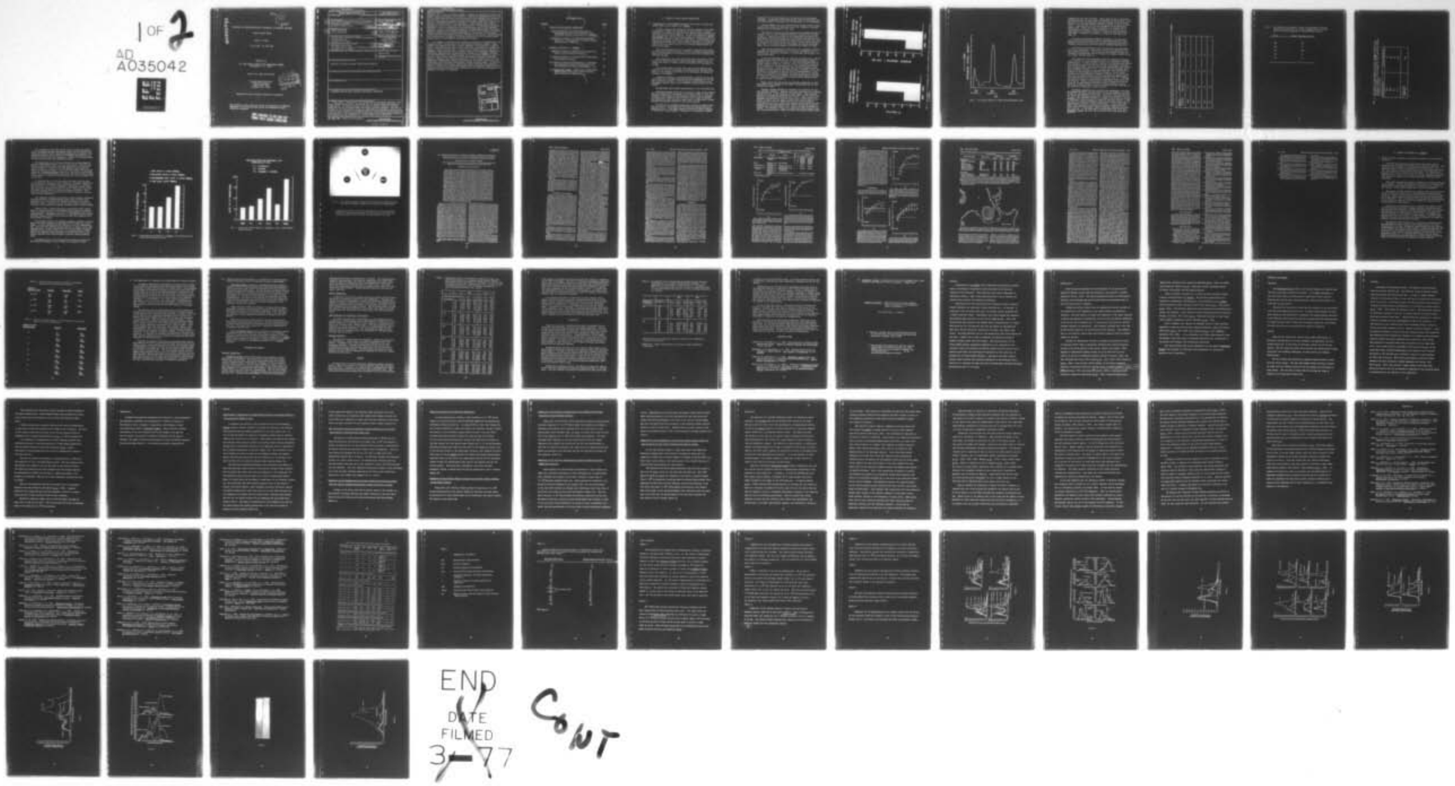
AD-A035 042 OHIO STATE UNIV RESEARCH FOUNDATION COLUMBUS F/G 6/13
ISOLATION AND CHARACTERIZATION OF PLASMODIAL AND BABESIAL ANTIG--ETC(U)
JAN 77 J P KREIER DADA-17-72-C-2072
UNCLASSIFIED OSURF-3365-3-REV-77 NL

1 OF 2
AD
A035042



END
DATE
FILMED
3-77

CONT

12

NW

ISOLATION AND CHARACTERIZATION OF PLASMODIAL AND BABESIAL ANTIGENS

Annual Summary Report

Julius P. Kreier

1 July 1976 - 30 June 1977

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D. C. 20314

Contract No. DADA-17-72-C-2072

The Ohio State University
Research Foundation
1314 Kinnear Road
Columbus, Ohio 43212

Approved for public release; distribution unlimited.

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

. UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER

2. GOVT ACCESSION NO.

3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle)

6 ISOLATION AND CHARACTERIZATION OF PLASMODIAL AND BABESIAL ANTIGENS.

5. TYPE OF REPORT & PERIOD COVERED

9 Annual rept.
1 Jul[redacted]76 — 30 Jun[redacted]77

6. PERFORMING ORG. REPORT NUMBER

14 OSURF-3365-3—Rev[redacted]-77

7. AUTHOR(s)

10 Julius P. Kreier

8. CONTRACT OR GRANT NUMBER(s)

15 DADA-17-72-C-2072

9. PERFORMING ORGANIZATION NAME AND ADDRESS

The Ohio State University Research Foundation
1314 Kinnear Road
Columbus, Ohio 43212

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS

12 73p.

11. CONTROLLING OFFICE NAME AND ADDRESS

Department of the Army
U.S. Army Medical Research & Development Command
Washington, D. C. 20314

12. REPORT DATE

11 15 Jan[redacted]77

13. NUMBER OF PAGES

64

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)

15. SECURITY CLASS. (of this report)

Unclassified

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Plasmodia; Babesia; Host Parasite Interaction; Immunology

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report is divided into two separate but interrelated subsections; the first concerns host-parasite interaction and the second antigen isolation and immunization. We proposed several years ago to identify protective antigens among the variety of antigens which the malaria parasite produces. This was to be done by the absorption of protective serum with free parasites and with fractions of free parasites and by immunization with free parasites and fractions of free parasites. The demonstration and characterization of macrophage-cytophilic and parasite coating antibodies is a direct result of our efforts to

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

267 360

Block 20 (Con't.)

determine why it was not readily possible to remove the protective factors from immune serum by absorption with parasites. What we have determined is that one of the complicating factors is the low avidity of the antibody which attaches to the parasites, and that a second and perhaps even more important factor is the role of a macrophage-cytophilic antibody in the immune serum which mediates attachment of the parasites to the macrophages. This antibody must attach to macrophages before it will react with the parasites. The macrophage-cytophilic antibody is an IgG_1 antibody while the antibody which binds to the parasites directly is IgG_2. The macrophage-cytophilic antibody may be eluted from macrophages by heating the sensitized macrophages at 56°C for 30 minutes or purified from hyperimmune serum by column chromatographic techniques. We have demonstrated that the macrophage-cytophilic antibody and the parasite coating antibody act synergistically in vivo to bring about destruction of the parasites while alone neither is very effective in protecting the host.

Our attempts to identify which antigens are protective by immunization have progressed more as planned than have the antibody absorption studies. These studies have demonstrated that a soluble component of the parasite is significant in inducing immunity, and we have tentatively identified this soluble component as surface coat material. In our work on identification of protective antigens we have obtained some preliminary results from experiments designed to facilitate identification of stage specific antigens in P. berghei parasites. We have demonstrated that freeze-thawing of free parasites releases at most 1/5 to 1/3 of the parasite material and that significant protection stimulating material remains in the insoluble components. We have demonstrated that a new technique, cryoimpacting, solubilizes practically the entire parasite and this observation will facilitate fractionation studies already planned. We also have some preliminary results which confirm that density gradient centrifugation is useful in separation of parasitized erythrocytes by development stage of the contained parasite and that this same technique will be useful in separating free parasites by stage of development.

TABLE ~~OF~~ CONTENTS;

A. STUDIES OF HOST-PARASITE INTERACTION

I. A demonstration of macrophage-cytophilic and parasite coating antibodies found in rats immune to P. berghei.

We have observed that incubation of freed parasites with immune serum does not reduce the infectivity of the parasites, and that protection is achieved in vivo only when immune serum was added along with the antibody coated parasites. We have also found that Plasmodium berghei free parasite survival in vivo, as indicated by the onset of parasitemia in a fully susceptible host, is independent of the duration of in vitro incubation in immune serum prior to intravenous innoculation into the host. These results indicated to us that the mode of action of immune serum is not by direct neutralization of parasites in vitro.

The work presented here was an attempt to determine the nature of this in vivo protection which involves a fraction of immune serum which is not adsorbed to the parasite, but which does facilitate destruction in vivo.

Our recognition that some factor other than the parasite itself in the host-parasite system is affected by immune serum led us to examine the effect of immune serum on macrophages both in vitro and in vivo.

We considered that the most likely way in which immune serum could act on the host was through the involvement of specific recognition of the parasite facilitated by the attachment of cytophilic antibody to macrophages.

Cytophilic antibody is an immunoglobulin, primarily of the IgG class, which has a stronger affinity for the macrophage than it does for its specific antigen. After cytophilic antibody binds to the macrophage, however, there is an increased binding affinity for the antigen.

We approached this problem experimentally in the following ways.

Peritoneal washout cells were collected in tissue culture Medium 199 and incubated for ten minutes in Tris NH_3Cl buffer to lyse erythrocytes, then washed and re-suspended in Medium 199 and incubated in plastic Petri dishes at 37°C in 5% CO_2 for at least 30 minutes. Any non-adherent cells were then washed away with Hanks BSS, leaving primarily macrophages adherent to the culture dishes.

To determine if cytophilic antibody is present in rodents which have had malaria, hyperimmune CDF rat serum was precipitated with ammonium sulfate. This gamma globulin fraction was then conjugated to fluorescein isothiocyanate. Monolayers of unstimulated, normal

peritoneal CDF rat macrophages were reacted with this hyperimmune conjugate. A population of macrophages was found to selectively attach the conjugate, resulting in the fluorescence of the macrophages.

The macrophages were also sensitized with either normal or hyperimmune serum, washed and then used to test for cytophilic antibody mediated parasite adherence in the cold.

The effects of arming the macrophages with macrophage-cytophilic antibody prior to their incubation with free parasites can be seen in Fig. 1. Twenty-five percent of the macrophages pretreated with hyperimmune serum and then washed were associated with adherent parasites as opposed to only 9% of those pretreated with normal rat serum. Of the actual parasites counted, more than 3 times as many parasites were found to be associated with macrophages pretreated with hyperimmune serum as with macrophages pretreated with normal rat serum.

Once having established the presence of the cytophilic antibody, isolation and characterization of the specific immunoglobulin responsible for cytophilic attachment to macrophages and parasite coating was undertaken.

A pool of hyperimmune CDF rat serum was fractionated on a QAE-50 Sephadex column, and fractions were eluted with ethylene diamine acetate buffer at pH 7.2, 5.0 and 4.0. The eluate was monitored by optical density at 280 nm. Fraction 1 was in the washout volume at pH 7.2, Fraction 2 was eluted at pH 5.0, and Fraction 3 was eluted at pH 4.0 (Fig. 2). These fractions were tested by immunoelectrophoresis for identification and purity assay. Fractions 1 and 2 were identified as belonging to the IgG class of immunoglobulins. Fraction 1 was determined to belong to the subclass IgG_1 as its electrophoretic character is more cathodic than the IgG_2 globulin, Fraction 2. Fraction 3 consisted primarily of IgM and other serum proteins. No IgG was found in Fraction 3.

Each fraction was then tested for specific reactivity to fixed blood smears of *P. berghei* and to normal, unstimulated CDF rat peritoneal macrophages.

Parasite coating antibody was assayed by the indirect fluorescent antibody technique. Antigen slides were prepared from *P. berghei* infected CDF rats on the third day of parasitemia. There antigen slides were treated with dilutions of HIRS, NRS, and the three serum fractions and incubated for 30 minutes at 37°C. After washing with phosphate buffered saline (pH 7.2) slides were reacted with 1:4 dilution of rabbit anti-rat gamma globulin-fluorescein conjugate. After washing in P.B.S., slides were mounted in 10% PBS Glycerol and examined on a Zeiss microscope using exciter filters, EG 12 and 38. Fluorescence was graded from 0 to 4+, with 4+ having the maximum intensity. The parasites treated with hyperimmune serum showed a typical fluorescent pattern. Mature schizonts fluoresced brightly while

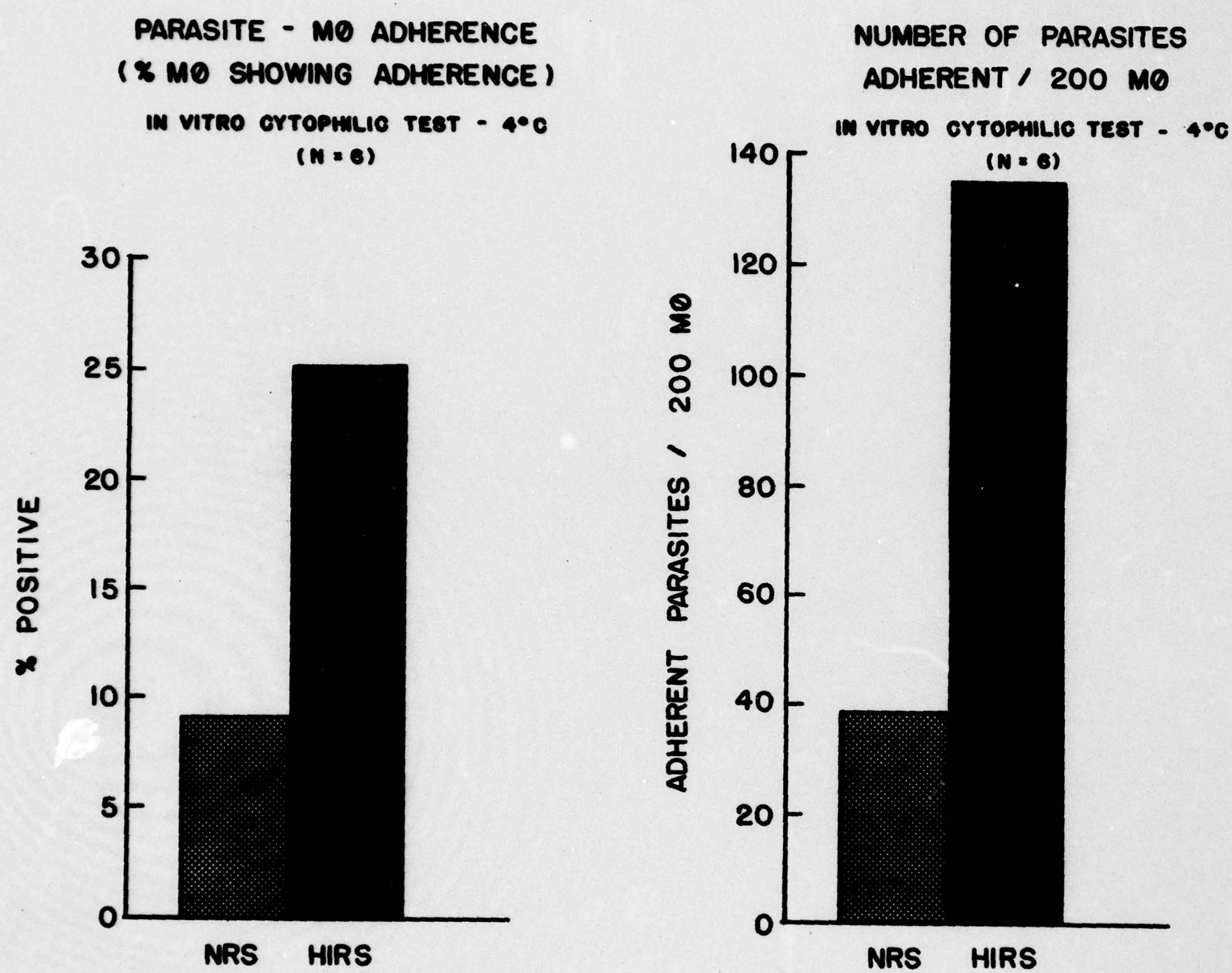


Fig. 1. The effects of macrophages with cytophilic antibody upon their ability to retain free <u>P. berghei</u>.

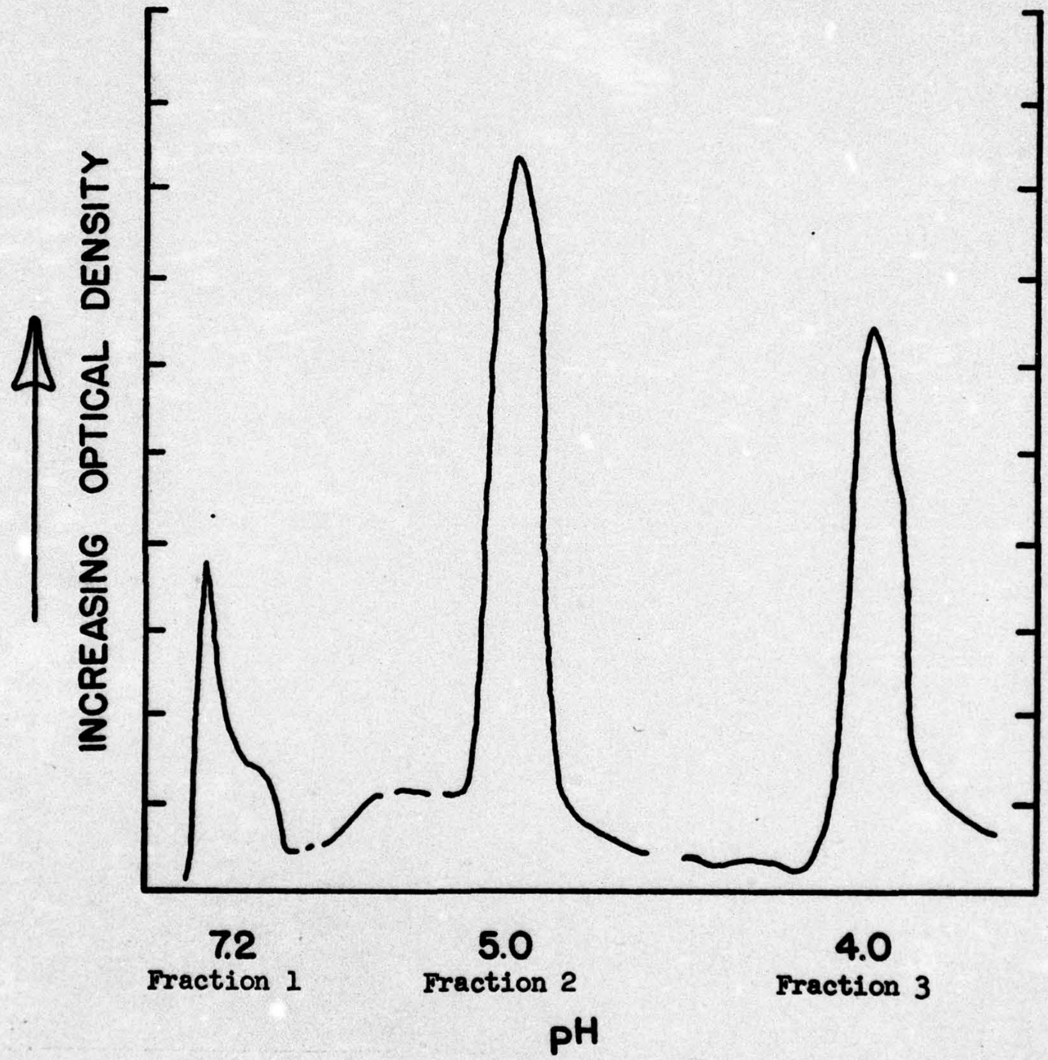


Fig. 2. pH elution pattern of HIRS from QAE-Sephadex A-50.

trophozoites were less brilliant. There seemed to be a correlation between intensity of fluorescence and maturity of the parasite. This may be due to the development of specific antigens as the parasite matured. The parasite coating antibody was found to reside in Fraction 2. Antibody titers in F-2 are comparable to unfractionated immune serum. Minor reactivity in Fraction 1 is probably due to heterogeneity of binding. Minor reactivity in F-3 may be due to minor parasite specificity in IgM or it may be due to nonspecific attachment. Reactivity in Fraction 3 is only in indiluted serum (Fig. 3).

To test for macrophage-cytophilic antibody in the fractions, normal unstimulated peritoneal CDF rat macrophage monolayers were exposed to the serum or fractions and then assayed by the indirect antibody technique and by the fluorescent antibody blocking test.

The percent of fluorescent cells in the total cell population present was determined. Fluorescent cells with an intensity of 2+ were considered positive. The results of the indirect FA test for cytophilic antibody show that the cytophilic antibody resident in the F-1, (IgG_1) fraction (Fig. 4).

The location of the macrophage-cytophilic antibody in Fraction 1 was confirmed by a more sensitive assay: the fluorescent antibody blocking test. In this procedure serum and its fractions were reacted with macrophages for $\frac{1}{2}$ hour at 4°C, rinsed in cold Hank's Balanced Salt Solution (HBSS), then reacted with the hyperimmune serum fluorescein conjugate for $\frac{1}{2}$ hour at 4°C. In this test the unlabeled cytophilic antibody attaches to the macrophage and blocks subsequent attachment of FITC-conjugated cytophilic antibody. By the direct staining with FITC-conjugated cytophilic antibody it was shown that 57% of the macrophages present in the particular macrophage population used were able to bind cytophilic antibody (Fig. 5). Blocking activity was present in unfractionated hyperimmune serum and in the F-1 fraction. Normal rat serum had a little blocking activity possibly due to some cytophilic antibody normally present in serum. The minor blocking activity in the F-2 fraction may have been due to the presence of some IgG_1 in this fraction.

Cytophilic antibodies were adsorbed from hyperimmune serum onto macrophage monolayers, and recovered from the macrophages by elution at 56°C for 30 minutes in Hank's BSS. The eluate, the absorbed serum and normal and hyperimmune serum were tested for protective activity _in vivo_. This procedure allowed us to compare the protective activity of hyperimmune serum depleted of cytophilic antibody activity, to the activity of a purified cytophilic antibody-containing eluate, and to the protective activity of the original serum. In one test hyperimmune serum depleted of cytophilic antibody by being adsorbed 6 times with 5×10^8 macrophages, showed a moderate reduction in protective activity. On the other hand six adsorptions with 5×10^8 free _Plasmodium berghei_ parasites per ml did not reduce the protective activity.

Fig. 3. Indirect FA test for antiplasmodium berghei antibodies in hyperimmune rat anti-*P. berghei* serum and fractions prepared on QAE-50 Sephadex columns.

Type of Serum or Fraction	Degree of Fluorescence at Serum or Fraction Dilution Shown		
	Undiluted	1/16	1/32
HIRS	4+	3+	3+
NRS	0	0	0
F-1	2+	+/-	0
F-2	4+	3+	3+
F-3	1+	0	0

Fig. 4. The results of an indirect FA test for macrophage-cytophilic antibody in hyperimmune rat anti-*P. berghei* serum or in fractions of the serum prepared in QAE-50 Sephadex columns.

Serum	Percent fluorescent cells
IRS	52%
NRS	0
F-1	12%
F-2	0
F-3	0

Fig. 5. The ability of hyperimmune rat anti-*P. berghei* serum and its fractions prepared on QAE-50 Sephadex columns to block direct fluorescent staining of macrophages by FITC labeled macrophage-cytophilic antibody.

Blocking Serum	% Cells Fluorescing
HIRS	1%
NRS	33%
F-1	2%
F-2	42%
Direct FAT: HIRS-FITC	57%

In a subsequent test (Fig. 6) the relative protective effects of the macrophage adsorbed hyperimmune serum, and the eluated antibodies recovered from the adsorbing macrophages were compared. The macrophage adsorbed serum suffered a substantial reduction in protective activity, while the cytophilic antibody in the eluate showed no protective activity when administered alone.

The hyperimmune serum fractions obtained by elution chromatography on QAE-Sephadex A-50 at pH 7.2, 5.0, and 4.0. (Fig. 2) were tested in vivo against normal and hyperimmune serum for protective activity (Fig. 7). Weanling rats were medicated with normal rat serum, or protective serum, or serum fractions, before the challenge innoculum of Plasmodium berghei was given. Any delay in parasitemia beyond the value obtained with normal serum was considered as evidence of protection. We used the 1% parasitemia level as an arbitrary reference point.

As shown in Fig. 7, when these fractions were tested in vivo it was found that Fraction 1 had virtually no protective value alone, whild Fraction 2 contained moderate protective activity. However, when Fractions 1 and 2 were recombined, a synergistic effect resulting in greatly increased protection occurred. Fraction 3 was found to contain minimal amounts of parasite-coating antibodies. Both normal and hyperimmune rat serum were included as negative and positive controls respectively.

The cytophilic antibody and parasite coating antibody containing fractions obtained from QAE-Sephadex columns and by elution from macrophage were also examined by immunoelectrophoresis and double diffusion in gel. Reactivity of all the immune serum fractions was verified by the fluorescent antibody technique prior to their testing.

Figure 8 shows a double-diffusion in gel pattern which reveals lines of identity between the macrophage-eluted cytophilic antibody and the antibody isolated in Fraction 1 by QAE-Sephadex A-50 elution chromatography. QAE-Sephadex Fraction 2, containing parasite-coating antibody, produced several lines, none of which were identical with the cytophilic antibody.

Thus we have isolated a macrophage-cytophilic antibody in malaria immune rats which promotes the adherence of free parasites to macrophages. We have characterized this antibody as an IgG_1 immunoglobulin. The well-known parasite-coating antibody is shown to be an IgG_2 immunoglobulin. We have demonstrated that the macrophage-cytophilic antibody is nonprotective alone, but acts synergistically in combination with parasite coating antibody to increase the level of protection afforded above that provided by parasite-coating antibody alone.

The demonstration of this dual antibody system in the host defense against plasmodia, if it is found to be present in other

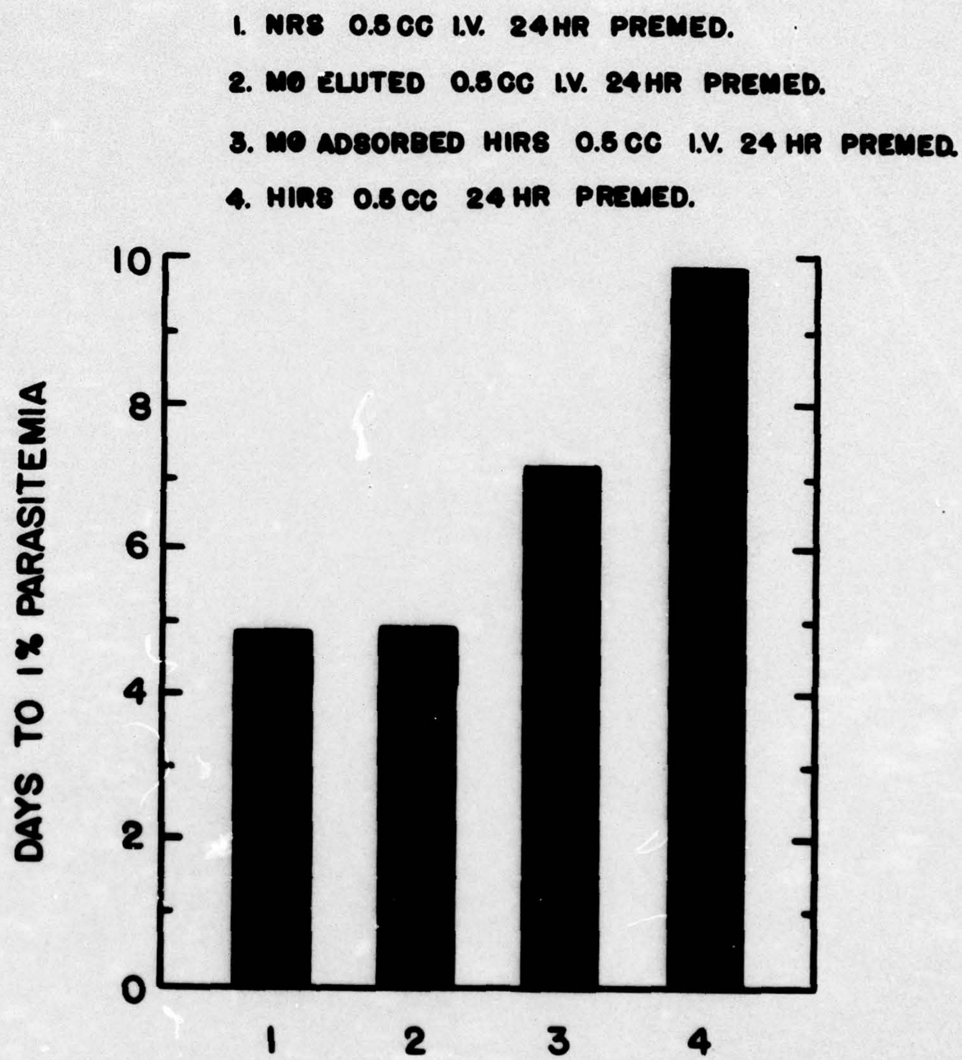


Fig. 6. Protective effects against P. berghei of MO absorbed and MO eluted HIRS fractions in rats.

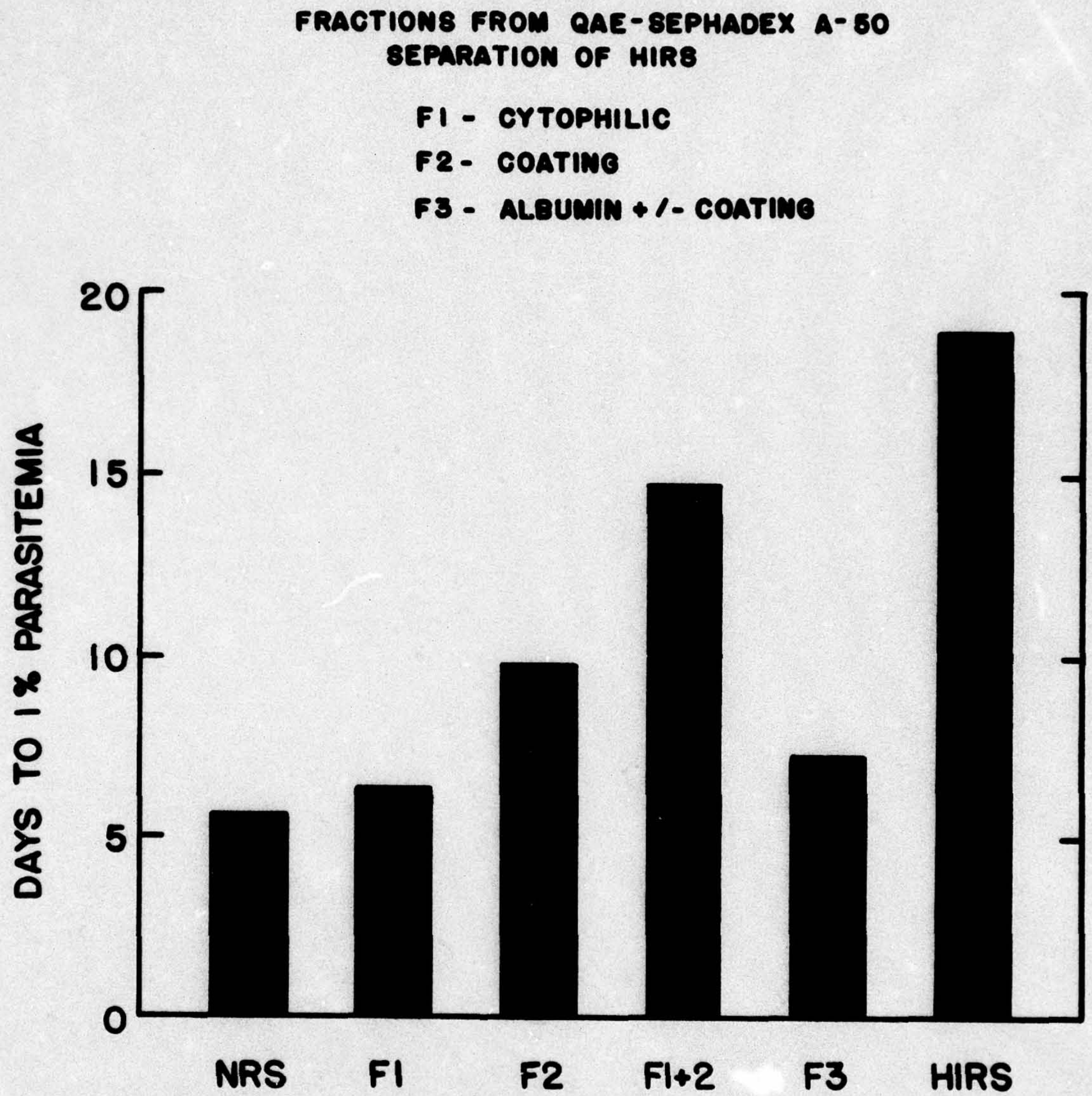


Fig. 7. Protective effects against P. berghei in rats of QAE Sephadex HIRS-fractions.

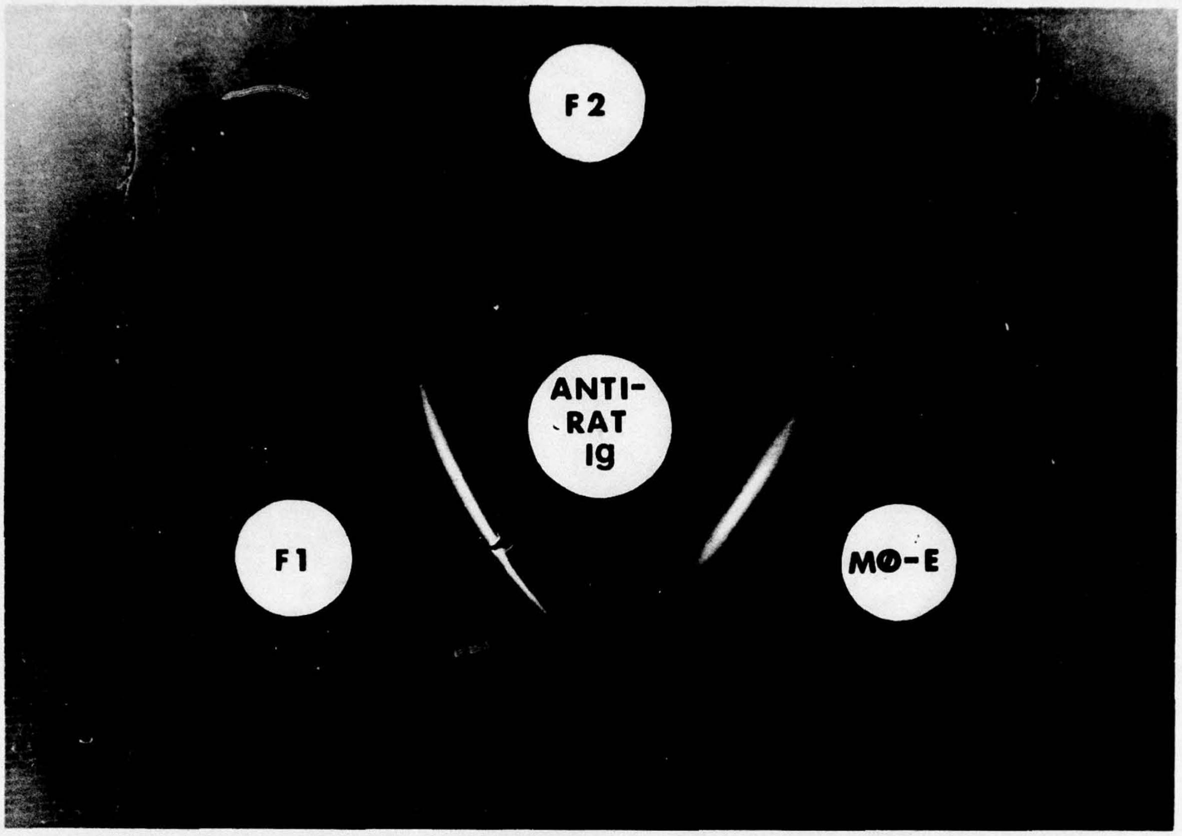


Fig. 8. Gel diffusion pattern obtained with fractions of $HIRS^V$ obtained from QAE 50 Sephadex columns and by elution from macrophages.

plasmodial systems, would aid in explaining the poor correlations which have been observed between antibody levels in the serum and protection which can be conferred by administration of serum.

Vol. 14, No. 6
Printed in U.S.A.

II. Surface Properties of Extracellular Malaria Parasites: Electrophoretic and Lectin-Binding Characteristics

THOMAS M. SEED[1]* AND JULIUS P. KREIER

Blood Research Laboratory, American National Red Cross, Bethesda, Maryland 20014; and Department of Microbiology, Ohio State University, Columbus, Ohio 43210

Received for publication 12 July 1976

The surface charge and lectin-binding capacity of isolated malaria parasites and host erythrocytes were analyzed and compared by chromatographic, electrophoretic, and cytochemical methods. Results indicated that at physiological pH values both freshly prepared and glutaraldehyde-fixed parasites and erythrocytes possess a net negative surface charge. Both cell types were strongly bound to cation-exchange resins and underwent cathode-directed electrophoretic migration. The isoelectric points for erythrocyte-free parasites and uninfected erythrocytes were approximately 3.0 and 4.0, respectively. The different effects of selective enzymatic digestion and solvent extraction on the electrophoretic mobilities of free parasites and erythrocytes suggested that the chemical constituents responsible for the net negative surface charges on each type of cell are different. The surface charge of the free parasites seemed mainly to be a function of ionized phospholipids rather than of the ionogenic sialic acid moieties, which are the major contributors to the negative charge on erythrocytes. Results of lectin-binding studies indicated that specific glycosidic moieties (i.e., glucose, galactose, mannose, and *n*-acetylglucosamine), common to the erythrocyte surface, were either absent or in low concentration at the parasite's surface. These observations suggest that the normally intracellular malaria parasites have surface characteristics, differing from those of the host cell, characterized by a scarcity of lectin-binding receptors and sialic acid residues and by the major contribution of lipids to their surface charge.

Obligate intracellular parasitism is a biological phenomenon whose fundamental nature remains, for the most part, to be explained. It has been suggested that the intracellular parasitic habit exists because of the restrictive metabolic limitations imparted by inherently deficient limiting membranes (15). Not a great deal is known about the structure and function of limiting membranes of obligate intracellular parasites; this is especially true of the malaria parasites. Technical difficulties encountered in the isolation of intracellular parasites from infected host cells have been the major stumbling block. Recent advances in this area have included the development of improved isolation techniques (18-20) and the application of new cytochemical procedures (1, 6, 22, 23; T. M. Seed, M. Aikawa, and C. Sterling, Fed. Proc. 32:882, 1973) that circumvent the isolation problem. As a result, a number of recent studies suggest that significant compositional, structural, and functional differences exist between limiting membranes of intracellular parasites and their host cells. Rock and his colleagues (8, 20) reported finding significant differences in phospholipid composition between isolated plasma membranes of the two cell types. Parasite membranes were found to contain greater amounts of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol than erythrocyte membranes, but lesser amounts of sphingomyelin and phosphatidylserine. Similarly, differences in the spatial arrangement of surface phospholipids between the two cell types have been detected by cytochemical electron microscopy (23; Seed et al., Fed. Proc. 32:882, 1973). Major differences in surface carbohydrates also have been reported. Sialic acid, a major surface component of the erythrocyte membrane with functional ionogenic and antigenic properties, is either in low concentration or possibly entirely absent from the parasite's plasmalemma (22, 23).

Functional differences between erythrocyte membranes and those of plasmodia, as well as compositional ones, are now becoming evident. Sherman and Tanigoski (27-29) reported finding simple diffusion to be the major mechanism

[1] Present address: Division of Biological and Medical Research, Argonne National Laboratory, Argonne, IL 60439.

by which free amino acids entered isolated erythrocyte-free malaria parasites. Only five of the 17 amino acids tested gained entry into the parasite via active processes. In contrast, the majority of amino acids tested entered the erythrocyte by mediated processes. Such transport studies, together with the extracellular cultivation studies by Trager at The Rockefeller University which indicate that extracellular growth and reproduction are strictly dependent on the presence of normally nonpenetrating polar metabolities such as adenosine triphosphate, coenzyme A, nicotinamide adenine dinucleotide, etc. (32), tend to support the hypothesis that intracellular parasitism is selectively advantageous to these microbes since intracellular parasites can accumulate essential metabolities with only a minimal expenditure of energy (15).

The work presented here is an extension of our previous studies in which we attempted to characterize comparatively the surface structure of the obligate intracellular malaria parasite and that of the host cell. In this paper we evaluate the ionogenic properties and the lectin-binding capacities of the surfaces of the two cell types.

MATERIALS AND METHODS

General. A strain of *Plasmodium berghei* obtained from the Walter Reed Army Institute of Research was maintained by weekly syringe passage of infected blood in albino mice or young rats. When heavily parasitized blood samples were needed for the studies conducted, rats were injected intraperitoneally with *P. berghei*-infected rat erythrocytes. When the rats had obtained 40 to 50% parasitemia, they were anesthetized with ether and bled to death by cardiac puncture with syringes containing Alsever solution. The blood collected in Alsever solution was washed three times in phosphate-buffered saline (PBS) and run through a column filled with filter paper powder to remove the leukocytes, and the washed erythrocytes were prepared as a 10% suspension. This suspension was sonicated in a continuous-flow sonic oscillator (18, 19). The lysed erythrocytes were collected, centrifuged at 2,500 rpm (700 × *g*) for 5 min to remove unbroken erythrocytes, and then centrifuged at 7,500 rpm (6,800 × *g*) for 10 min to bring the parasites down. The parasites were resuspended in PBS and used within the next 24 h.

The parasites not used fresh were fixed in 1.25% glutaraldehyde overnight in the refrigerator. The next day the fixed parasites were washed three times in PBS and stored in the refrigerator until needed.

Ion-exchange procedures. The ion-exchange and molecular-sieve columns were prepared by the standard procedures recommended by the manufacturers. The various Sephadex resins were obtained from Pharmacia Fine Chemicals, Inc. (Piscataway, N. J.), and the diethylaminoethyl and Ecteola were obtained from General Biochemicals (Chagrin Falls, Ohio). The parasite preparations prepared by sonication of infected blood were passed through the columns by techniques similar to those used by Lanham and Godfrey (12) and Al-Abbassy et al. (2) for separation of trypanosomes from infected rat blood and culture fluids.

Lipid extrction procedure. Lipid-soluble materials (e.g., phospholipid and cholesterol) were extracted by techniques previously described (7, 16, 30). The cells were fixed with 1.25% glutaraldehyde overnight and then washed three times with PBS. The fixed cells were placed in a large test tube containing a 2:1 chloroform-methanol solution (25 ml of methanol, 50 ml of chloroform) and 2 to 5 ml of cells in saline. The tube was chilled on ice and shaken for 1 min. The phases were allowed to separate, and the parasites were drawn off with the methanol phase, packed by centrifugation, and placed in another test tube containing a fresh 2:1 chloroform-methanol solution. The extraction was repeated five to six times. After the second or third treatment the phases did not separate; therefore, after mixing and waiting 5 to 10 min, the solution with the parasites or erythrocytes was centrifuged, and then the cells were resuspended in the chloroform-methanol solution; the above procedure was repeated. After the lipid was extracted, the parasites or erythrocytes were washed three times in methanol to remove the chloroform and then three times in saline to remove the methanol.

Trypsin treatment. The PBS-washed erythrocytes or parasites were suspended to a 10% concentration in a freshly prepared 0.25% trypsin solution in phosphate-buffered physiological saline solution, pH 7.2, and incubated for 1 min in a 37°C water bath. After digestion, the cells were washed by centrifugation in phosphate-buffered physiological saline and suspended in the same solution for examination.

Whole-cell electrophoresis. Electrophoretic mobilities of intact cells were measured utilizing a Zeiss Cytopherometer, equipped with platinium electrodes. Cell mobility testing was done in the following manner. The electrophoresis chamber was filled and allowed to equilibrate for 15 min with the appropriate pH buffer. A few drops of the sample to be tested were placed in the funnel and drained into the chamber. The current was switched on and set at 20 mA. A stopwatch was used to measure the time one cell took to travel past three vertical lines of the reticule. Then the polarity was reversed and the same cell was measured again, traveling in the opposite direction. Twenty to 30 cells were measured at each pH for each cell type. After each run, the buffer was drained and the chamber was washed out with distilled water several times before the next pH buffer was added.

The migrational velocity (v) of each cell in the field was determined by the equation $v = L/T$, where L equals the distance (in micrometers) the cell traveled and T equals the time in seconds. The average velocity (in micrometers per second) was determined for all the cells measured at a given pH. The mobility of the cell was determined by the equation $M = v/$

E, where M is the mobility, v is the average velocity of the cell, and E is the electric field strength of the system. (Field strength is a function of the amperage times the specific resistance of the suspending media divided by the cross-sectional area of the electrophoresis chamber.) Cell mobilities (M), expressed in terms of distance traveled micrometers per second per volt per square centimeter, were plotted on a graph against pH. Isolectric points were determined by direct examination of these pH mobility curves.

The buffer system used was the Universal Buffer (Marine Biological Laboratory, 1956), which extended from pH 2 to 11. The predominant ions present were phosphate, citrate, and borate. The buffers were made isotonic by the addition of 0.85% NaCl. Buffers were made up fresh before each run and stored in a refrigerator.

Lectin-binding studies. Lectin-binding studies (i.e., slide agglutinations and cytochemical electron microscopy) were carried out according to procedures similar to those described in an earlier study (25).

(i) Lectins. Purified concanavalin A (Con A) agglutinin was purchased from Miles Laboratories, Inc. Wheat germ agglutinin was purified from wheat germ lipase (Sigma Chemical Co.) by affinity column chromatography (13). Other lectins, *Lathyrus sativus*, *Ricinus communis*, *Agaricus biporus*, *Phaseolus coccineus*, and *Robinia pseudoaccia*, were kindly supplied by G. A. Jamieson. All of the lectins utilized exhibited highly specific biological activity in platelet agglutination assays (10).

(ii) Slide agglutination assays. Erythrocytes and free-parasite isolates were washed in PBS as described above. Cells were initially fixed in 1.25% glutaraldehyde-4% sucrose-0.05 M phosphate buffer (pH 7.2) and then thoroughly washed in PBS. The cell samples were then resuspended in PBS to a final concentration of 2.5×10^7 cells/ml. Ten microliters of each cell suspension was mixed by mechanical rotation on a microscope slide with 10 μl of each of the serially diluted (twofold dilutions) lectin concentrates (2 mg/ml). After a 1-min mixing period, the droplets were covered with glass slips and sealed with vaseline to prevent drying during the subsequent 30-min incubation at 25°C. The extent of cell agglutination was determined visually by light microscopy.

The agglutination reactions were controlled either by omitting the lectin entirely or by serially diluting the lectins in PBS containing specific sugar inhibitors at 0.1 M concentrations. D-Glucose was used to inhibit Con A and *L. sativus* agglutinations, whereas galactose was used to inhibit *R. communis*, *A. biporus*, and *R. pseudoaccia* reactions. *N*-acetylglucosamine was used as an inhibitor for wheat germ lectin. D-Galactose was used as the inhibitor for *P. coccineus*-mediated agglutinations, although the active sugar determinant has not yet been determined (10).

(iii) Cytochemical electron microscopy. Aliquots of erythrocytes and free parasites were fixed as previously described. Cell samples were washed either in PBS or in PBS containing a 0.1 M concentration of specific sugar inhibitor of lectin binding. Cells were then resuspended to a 50% packed-cell volume in a total 0.1 M concentration of the ferritin-lectin conjugate reagent for 30 min at 25°C. Samples were then rinsed twice in either the buffer alone or in a buffer-inhibitor medium. These treated samples were then postfixed, dehydrated, and embedded for thin-section preparation according to standard procedures (23).

The affinity column-purified plant agglutinins were conjugated to ferritin by a modification of the glutaraldehyde coupling method of Avrameas (4) and later Nicolson and Singer (17). Aliquots (10 mg) of ferritin (Nutritional Biochemicals Corp.) were mixed with 5-mg portions of each lectin suspended in 2 ml of a 0.5 M NaCl-0.05 M phosphate-buffered solution (pH 6.8) that contained a 0.1 M concentration of specific sugar inhibitor (e.g., 0.1 M glucose for Con A-ferritin coupling). Twenty microliters of a 3% glutaraldehyde solution (biological grade, Fisher Chemical Co.) was added to each vial with constant stirring. The mixtures were stirred for 1 h at 25°C, after which single drops of 0.1 M ammonium chloride were added to the samples to stop the reactions. The solutions were then dialyzed at 4°C against sugar-free 0.5 M PBS. After a 4- to 6-h dialysis period, the reaction mixtures were centrifuged at $2{,}000 \times g$ for 10 min. The supernatants were removed and stored at 4°C until needed. (Initially these preparations were chromatographed on BioGel columns; however, we found this step not to be essential for our experiments since we noted an almost complete inhibition of binding of the nonchromatographed lectin conjugates to reactive erythrocytes after the addition of specific sugars.)

RESULTS

Absorption-elution characteristics. Both isolated, erythrocyte-free malaria parasites (*P. berghei*) and rat erythrocytes were retained on various types of basic anion-exchange resins under a variety of test conditions (Table 1). Neither the parasites nor the erythrocytes could be eluted from the resins by flushing the resins with PBS at pH values between 6 and 8, by increasing the ionic strength of the saline flush from physiological levels to four times normal or by flushing the columns with physiological saline containing 4% amino acid hydrolysate. However, when the same mixtures were passed through columns containing an uncharged resin of the type that served as the base for the charged resin, neither the parasites nor the erythrocytes were retained (Table 1).

Electrophoretic characteristics of unmodified erythrocyte-free parasites. Preparations of fresh small *P. berghei* parasites migrated toward the anode in the particle electrophoresis system at all pH values above 4. The isoelectric point of the parasites was estimated to be in the region of pH 3 (Fig. 1).

Electrophoretic characteristics of chemi-

TABLE 1. *Absorption-elution characteristics of rat erythrocytes and free plasmodial parasites on Sephadex columns*

Type	Description	Eluent	No. of trials	pH	Results: Erythrocytes	Results: Parasites
DEAE-cellulose[a]	Strongly basic anion exchanger	PBS, physiological	2	6	Retained	Retained
			1	7	Retained	Retained
			2	7.6	Retained	Retained
			1	8	Retained	Retained
Ecteola	Weakly basic anion exchanger	PBS, physiological	5	7	Retained	Retained
DEAE-Sephadex A-25	Strongly basic anion exchanger	PBS, up to 4 times physiological strength	2	7	Retained	Retained
DEAE-Sephadex A-25 or Ecteola	Strongly basic anion exchanger; Weakly basic anion exchanger	PBS, physiological strength, plus 4% amino acid hydrolysate	2	7	Retained	Retained
Sephadex G-25	Uncharged base resin for A-25	PBS, physiological strength	1	7	Not retained	Not retained

[a] DEAE, Diethylaminoethyl.

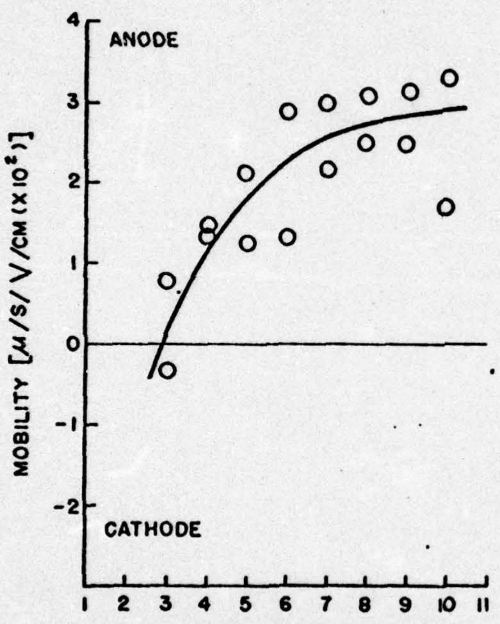


FIG. 1. *Relationship of pH to mobility of fresh sonically freed P. berghei parasites. At pH values above the isoelectric point of about 3, the parasites have a negative charge.*

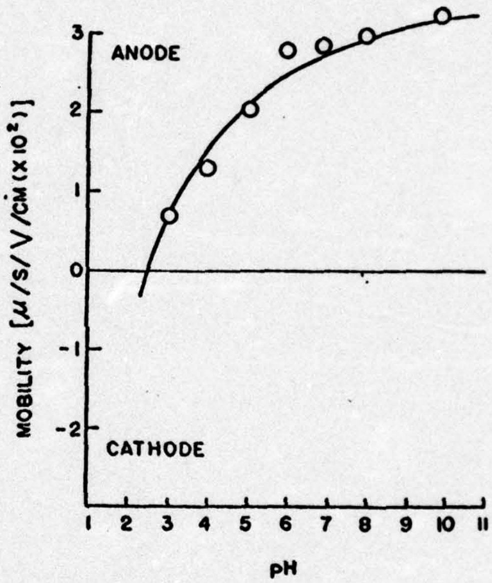


FIG. 2. *Relationship between pH and mobility of gluteraldehyde-fixed, sonically freed P. berghei. Gluteraldehyde fixation did not much affect the charge characteristics of the parasites, which indicates that amino and guanidinium groups do not contribute significantly to the charge characteristics of the parasites.*

cally modified erythrocyte-free parasites. Glutaraldehyde treatment did not cause any significant change in the electrophoretic characteristics of *P. berghei* parasites (Fig. 2). In contrast, lipid extraction of gluteraldehyde-fixed *P. berghei* yielded parasites with a very low mobility that did not vary with respect to pH. Lipid extraction of gluteraldehyde-fixed erythrocytes, however, failed to significantly alter the charge characteristics associated with unextracted erythrocytes (Fig. 3). Trypsin treatment under conditions that markedly reduced the charge of erythrocytes (Fig. 4) caused a moderate reduction in the charge of *P. berghei* (Fig. 5).

Lectin-binding capacity of erythrocytes and isolated parasites. Rat erythrocytes were agglutinated by six of the seven lectins tested at concentrations as low as 25 μg/ml (Table 2). Similarly, these agglutinating lectins, in the form of ferritin conjugates, bound specifically to the surfaces of intact erythrocytes and to eryth-

rocyte ghosts. These agglutinations and conjugate-binding reactions were shown to be selectively inhibited by small additions of lectin-specific sugars (Table 2). The exception to this was the *Phaseolus coccineus* lectin-induced reaction, whose sugar-binding determinant has not yet been determined (10). In contrast, all of the lectins tested failed to agglutinate, to any appreciable extent, isolated, erythrocyte-free parasites, although, cytochemically, a small percentage of parasites displayed a small amount of bound lectin-ferritin conjugate. However, most of this bound lectin conjugate was associated with the enveloping host cell membrane and not with the parasite's plasmalemma (Fig. 6). The Con A-ferritin conjugate was the only reagent that consistently showed some reactivity with the parasite's surface. Con A binding to the parasite's plasmalemma was slight compared with that of host erythrocytes and seemingly was not dependent on the stage of parasite development (i.e., merozoite, trophozoite, etc.).

DISCUSSION

In a parasitic infection such as malaria, in which the relationship of the erythrocyte and the parasite is so vitally important, the electro-

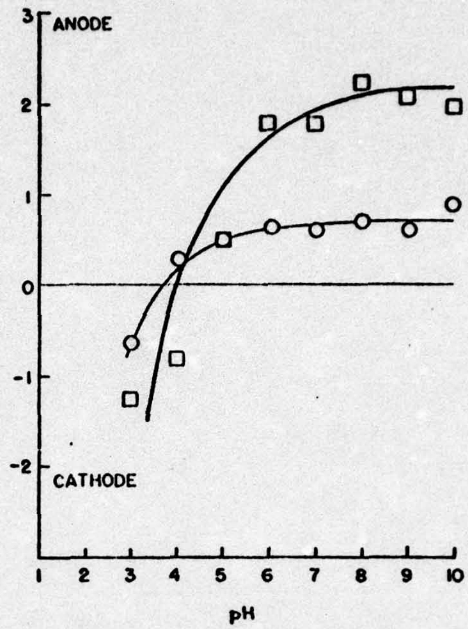


FIG. 4. *Effect upon mobility of erythrocytes of treatment with trypsin for 1 min at 37°C (○). The mobility of the treated cells is markedly reduced over a wide range of pH values, which indicates that the charged groups were removed by trypsin treatment. Mobilities of control erythrocytes represented by squares (□).*

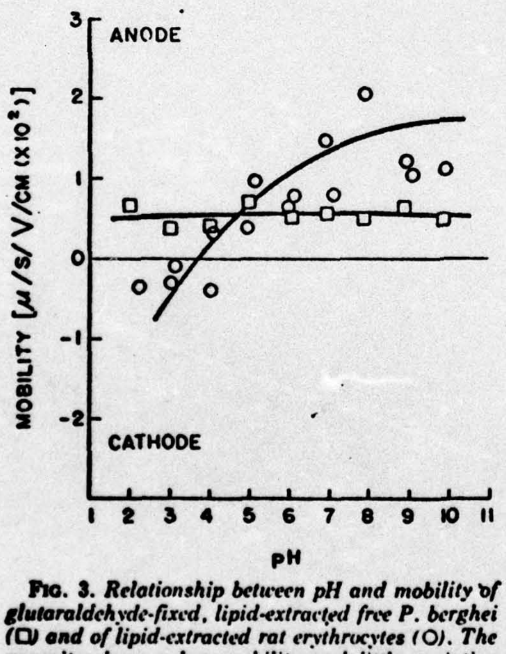


FIG. 3. *Relationship between pH and mobility of glutaraldehyde-fixed, lipid-extracted free P. berghei (□) and of lipid-extracted rat erythrocytes (○). The parasites have a low mobility and little variation with respect to pH, indicating that most of the charge on the parasite was removed by the lipid extraction. The rat erythrocytes have a fairly high isoelectric point (3.5). The mobility of the erythrocytes is within normal limits for glutaraldehyde-fixed cells.*

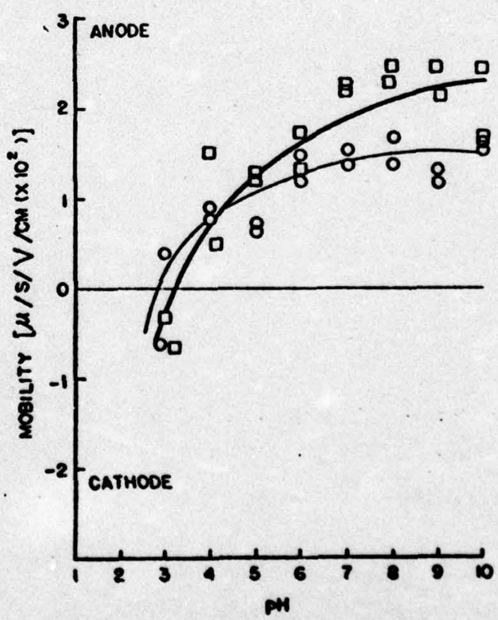


FIG. 5. *Effect upon the mobility of free P. berghei parasites of treatment with trypsin for 1 min at 37°C (○). The mobility of the treated parasites was slightly reduced as compared with contol parasites. (□).*

TABLE 2. *Summation of lectin- and iron colloid-binding capacity of erythrocyte-free Plasmodium berghei and rat erythrocytes*

Surface-active reagent	Sugar specificity	Erythrocytes		Free parasites	
		Agglut[a]	Conjug[b]	Agglut	Conjug
Plant lectins					
Wheat germ agglutinin	GlcNAc	Pos	Pos	Neg	Neg
Con A	Glc/Man	Pos	Pos	Neg	Pos (weak)
Lathyrus sativus	Glc/Man	Pos	Pos	Neg	Neg
Ricinus communis	Gal	Pos	Pos	Neg	Neg
Mushroom agglutinin	Gal	Pos	Pos	Neg	ND
Phaseolus coccineus	ND	Pos	Pos	Neg	Neg
Robinia pseudoaccia	Gal/GlcNac/Man	Neg	Neg	Neg	ND
Iron colloids[c]					
Acetic acid hydrosol	Neuraminic acid	NA	Pos	NA	Neg
Propionic acid hydrosol	Phospholipids	NA	Pos	NA	Pos (strong)

[a] Slide agglutination assays. Positive result (Pos) for agglutinin assays indicates that test cells agglutinated in a lectin concentration of 25 μg/ml or more. Negative results (Neg) indicate that test cells failed to agglutinate in lectin concentrations up to 2.0 mg/ml. NA, Not applicable; ND, no determination made.

[b] A positive notation (Pos) indicates that aldehyde-fixed cells bound, specifically, lectin-ferritin conjugate or iron colloid. A negative notation (Neg) indicates a lack of ferritin or colloid binding.

[c] From Seed et al. (22).

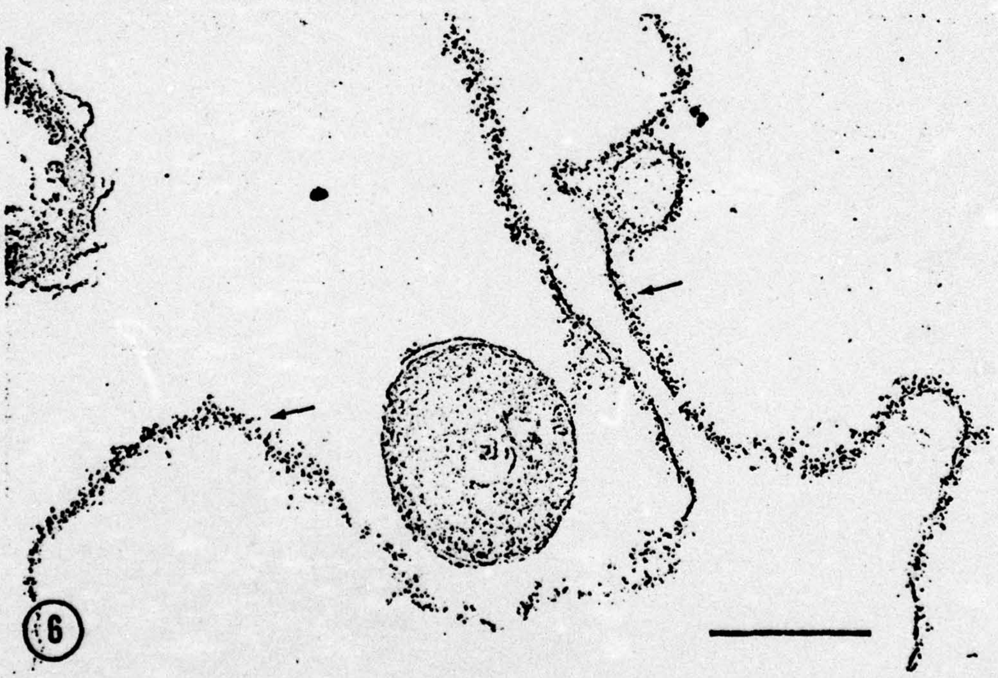


FIG. 6. *Electron micrograph of a mixture of free P. berghei parasites and erythrocyte membranes stained with wheat germ agglutinin conjugated with ferritin. The erythrocyte membranes (arrows) are heavily stained, but the parasite has little wheat germ conjugate attached. Line marker represents 0.5 μm. ×52,000.*

kinetic charge on the surface of the erythrocyte and parasite may be of considerable importance in such aspects of the infection as host specificity of the parasite, penetration of the erythrocyte by the parasite, and phagocytosis of the parasite by the host macrophages.

Recently a number of workers have attempted to evaluate, cytochemically, the ionogenic properties of the malaria parasites by using a positively charged iron colloid suspended in acetic acid (1, 6, 14, 22, 23; Seed et al., Fed. Proc. 32:882, 1973). Because of the low pH

(i.e., 1.8 to 3.0) at which the staining procedure is carried out, many surface components (e.g., amino acids) that act as weak acids are not ionized and, therefore, do not bind this type of colloid, whereas others, such as certain species of surface carbohydrates (e.g., sialic acid) do. It was observed that free parasites, in contrast to erythrocytes, bound little, if any, of the positively charged colloid (14, 22; Seed et al., Fed. Proc. 32:882, 1973) (Table 2). From this it was concluded that malaria parasites are less negatively charged than their host cells (14) and that this charge difference might be responsible for nonspecifically augmenting merozoite attachment to host cells, thus allowing more specific invasion-related processes to occur (1). From the results presented here, such hypothesized charge-dependent processes seem to be unlikely.

Seed and his colleagues (23), in subsequent work, confirmed the earlier cytochemical observation that the sialophilic iron colloid-staining procedure did not reveal exposed sialic acid groups at the parasite's surface such as those that occur on and give rise to the net negative surface charge on erythrocytes. Biochemical analysis supported the cytochemical observations by indicating that the isolated parasites contained only about one-half the amount of sialic acid per unit weight of erythrocytes. Using the same positively charged colloidal iron stain, Seed and colleagues further demonstrated that sialic acid was distributed randomly over the surface of leukocytes and platelets, as well as intact erythrocytes and erythrocyte ghosts (23).

These same workers also used a lipophilic iron colloid stain to localize lipid plaques at the surface of parasites and erythrocytes (23). The gross distribution of the plaques was somewhat similar for the two cell types; however, the parasites stained more intensely with lipophilic colloid than did erythrocytes (Table 2). The parasite's lipid distribution was characterized by the occurrence of large focal aggregates in a close-knit patchwork pattern, whereas the erythrocytes bound colloid to their surfaces in a thinner, more uniform pattern with much less colloid aggregation. Free parasites also exhibited an extremely thin mucopolysaccharide coat that was different from the thick glycocalyx layer of the erythrocytes. The sum of these observations indicated that the malaria parasite's membrane, with its high phospholipid and low carbohydrate content, is decidedly different from the limiting membranes of most mammalian cells. These differences in surface structure between parasite and host cell do not appear to extend to electrokinetic properties. Although the origins of surface charge for the two cell types appear different, the net negative charge of both cells is approximately the same. As Seed et al. (23) pointed out in an earlier paper, the failure of the malaria parasites to stain with a sialiophilic colloidal iron stain does not prove that parasites lack a net negative surface charge, only that they lack charge groups arising from neuraminic acid.

Our gel filtration and particle electrophoresis studies, in fact, indicate quite clearly that plasmodia have a net negative charge when suspended in solutions with a pH above about 3 and that they have a charge density not too dissimilar from that on the erythrocytes. The possibility that the retention of the parasites on the columns was due to the hydrophobic, lipid character of their membranes rather than to their charge was eliminated by the observation that the parasites were not retained on Sephadex G-25, which is the same as Sephadex A-25 except for the absence of the positively charged diethyl-(2-hydroxypropyl)aminoethyl groups.

It has been fairly clearly demonstrated by previous workers that intraerythrocytic *P. berghei* do not have significant amounts of *n*-acetyl neuraminic acid on their surfaces and that in fact their surfaces have relatively little exposed carbohydrate of any type (22). The lectin work reported in this paper supports the observation that the erythrocytic forms of *P. berghei* have little exposed carbohydrate. Further, it indicates that the parasite's surface lacks many specific glycosidic residues which commonly characterize many different types of cell surfaces and which, depending on the cell type, impart a wide variety of biological functions to the cell (i.e., antigenicity, etc.)

A number of studies have indicated that erythrocytic *P. berghei* are rich in phospholipid (8, 20, 23, 24). Since this material is ionic, it could serve as the charge-bearing vehicle for *P. berghei*. Since phosphatidylserine is the only phospholipid that carries a net negative charge as physiological pH values, it is assumed that this particular lipid species contributes significantly to the parasite's net negativity. This might seem somewhat contradictory to the previous biochemical work of Rock and his colleagues (8, 20), which indicated that phosphatidylserine, in contrast to phosphatidylcholine and ethanolamine, was in relatively low concentration compared with the quantity within the erythrocyte's plasmalemma. However, these biochemical data assess the total phosphatidylserine content within isolated membrane preparations, and not the relative distribution of these components within the membrane itself. It is presently recognized that

many vital membrane components are distributed asymmetrically within the bilayers of erythrocyte membrane (5, 26). Phosphatidylserine is one such component, localized primarily within the cytoplasmic side of the bilayer of the erythrocyte unit membrane (5). The reverse situation might exist regarding phosphatidylserine distribution within the parasite's plasmalemma, i.e., phosphatidylserine being localized primarily on the plasma rather than the cytoplasmic side of the bilayer. Such distributional asymmetry would concentrate the net negative charge-bearing phospholipid groups at the surface of the parasite. The erythrocyte also bears, under physiological conditions, a net negative charge that is due in large part to *n*-acetyl neuraminic acid on its surface (3; M. A. Madoff and E. H. Eylar, Fed. Proc. 20:62, 1961). Glutaraldehyde fixation does not modify the mobility of erythrocytes (11, 33), nor does a lipid extraction procedure that removes phospholipid (7, 31). Trypsinization does reduce the charge on erythrocytes, however, by cleaving the protein moiety of the *n*-acetyl neuraminic acid-bearing glycoprotein and thereby releasing the charged carbohydrate groups (20). In this work we observed that gluteraldehyde fixation does not modify the charge characteristic of *P. berghei*, but that extraction of phospholipid by procedures that do not affect the charge on erythrocytes abolishes the charge on *P. berghei*. We also observed that trypsinization does reduce the charge on *P. berghei* to some degree. From these observations one may conclude that a phospholipid is the major charge-bearing structure on erythrocytic *P. berghei* and that this lipid may be a part of a lipoprotein complex in the parasite's membrane.

ACKNOWLEDGMENTS

We wish to thank Joseph Hamburger for helping us prepare isolated parasites and Linda Burianek for her assistance in the mobility studies. We would also like to thank Grahm Jamieson for kindly providing us with samples of some of the purified lectins used in this study.

This work was supported in part by the U.S. Army Research and Development Command (DADA 17-72-C-2072) and by the American National Red Cross Blood Research Program (contribution no. 360).

LITERATURE CITED

1. Aikawa, M., and T. M. Seed. 1973. Cytochemical surface properties of malarial parasites enhancing penetration of host erythrocytes, p. 8. *In* Progress in protozoology, Fourth International Congress on Protozoology. Universite de Clermont, Clermont, France.
2. Al-Abbassy, S. M., T. M. Seed, and J. P. Kreier. 1972. Isolation of the trypomastigote form of *Trypanosoma cruzi* from a mixture of the trypomastigote and epimastigote forms of the parasite by use of a DEAE-cellulose column. J. Parasitol. 58:631-632.
3. Ambrose, E. J. 1965. Cell electrophoresis. J. and A. Churchill Ltd., London.
4. Avrameas, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. Immunochemistry 6:43-52.
5. Bretscher, M. S. 1972. Phosphatidyl-ethanolamine: differential labelling in intact cells and cell ghosts of human erythrocytes by a membrane-impermeable reagent. J. Mol. Biol. 71:523-528.
6. Cooper, G. W., and L. H. Miller. 1974. Propanoic acid-ferric oxide and hydrosols. Differential cell surface binding and its relation to membrane lipid. J. Histochem. Cytochem. 9:856-867.
7. Dawson, R. M. C., N. Hemington, and D. F. Lindsay. 1960. The phospholipids of the erythrocyte 'ghosts' of various species. Biochem. J. 77:226-230.
8. DeZeeuw, R. A., J. Wijsbeek, R. C. Rock, and G. J. McCormick. 1972. Composition of phospholipids in *Plasmodium knowlesi* membranes and in host rhesus erythrocyte membranes. Proc. Helminthol. Soc. Wash. 29:412-418.
9. Glaser, R. M., and H. C. Mel. 1966. Microelectrophoretic and enzymic studies concerning the carbohydrate at the surface of rat erythrocytes. Arch. Biochem. Biophys. 113:77-82.
10. Greenberg, J. H., and G. A. Jamieson. 1974. The effects of various lectins on platelet aggregation and release. Biochim. Biophys. Acta 345:231-242.
11. Heard, D. H., and G. V. F. Seaman. 1961. The action of lower aldehydes on the human erythrocyte. Biochim. Biophys. Acta 53:366-374.
12. Lanham, S. M., and D. G. Godfrey. 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-Cellulose. Exp. Parasitol. 25:251-521.
13. Marchesi, V. T. 1972. Wheat germ (*Tritium vulgaris*) agglutinin. Methods Enzymol. 28:354-356.
14. Miller, L. H., K. G. Powers, J. Finerty, and J. P. Vanderberg. 1973. Difference in surface charge between host cells and malaria parasites. J. Parasitol. 59:925-927.
15. Moulder, J. W. 1962. Biochemistry of intracellular parasitism. University of Chicago Press, Chicago.
16. Nelson, J. 1967. Lipid composition of erythrocytes in various mammalian species. Biochim. Biophys. Acta 144:221-232.
17. Nicolson, G. L., and S. J. Singer. 1974. The distribution and asymmetry of mammalian cell surface saccharides utilizing ferritin-conjugated plant agglutinins as specific saccharide stains. J. Cell Biol. 60:236-248.
18. Prior, R. B., and J. P. Kreier. 1972. Isolation of *Plasmodium berghei* by use of a continuous-flow ultrasonic system: a morphologic and immunological evaluation. Proc. Helminthol. Soc. Wash. 29:563-574.
19. Prior, R. B., and J. P. Kreier. 1972. *Plasmodium berghei* freed from host erythrocytes by a continuous-flow ultrasonic system. Exp. Parasitol. 32:239-243.
20. Rock, R. C., J. C. Standefer, R. T. Cook, W. Little, and H. Sprinz. 1971. Lipid composition *Plasmodium knowlesi* membranes: comparison of parasites and microsomal subfractions with host rhesus erythrocyte membranes. Comp. Biochem. Physiol. 38B:425-437.
21. Seaman, G. V.F., and D. H. Heard. 1960. The surface of the washed erythrocyte as a polyanion. J. Gen. Physiol. 44:251-268.
22. Seed, T. M., M. Aikawa, and C. R. Sterling. 1973. An electron microscope-cytochemical method for differentiating membranes of host red cells and malaria parasites. J. Protozool. 20:603-605.
23. Seed, T. M., M. Aikawa, C. Sterling, and J. Rabbege. 1974. Surface properties of extracellular malaria parasites: morphological and cytochemical study. Infect. Immun. 9:750-761.
24. Seed, T. M., and J. P. Kreier. 1972. *Plasmodium galli-*

naccum: erythrocyte membrane alterations and associated plasma changes induced by experimental infection. Proc. Helminthol. Soc. Wash. 39:387-411.

25. Seed, T. M., J. R. Seed, and D. Brindley. 1976. Surface properties of bloodstream trypanosomes (*Trypanosoma brucei brucei*). Z. Tropenmed. Parasitol. 27:202-212.
26. Sheetz, M. P., and S. J. Singer. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. Proc. Natl. Acad. Sci. U.S.A. 71:4457-4461.
27. Sherman, I. W., and L. Tanigoski. 1972. Incorporation of ^{14}C-amino acids by malaria (*Plasmodium lophurae*). V. Influence of antimalarials on the transport and incorporation of amino acids. Proc. Helminthol. Soc. Wash. 39:250-260.
28. Sherman, I., and L. Tanigoski. 1974. Incorporation of ^{14}C-amino acids by malarial plasmodia (*Plasmodium lophurae*). VI. Changes in the kinetic constants of amino acid transport during infection. Exp. Parasitol. 35:369-373.
29. Sherman, I. W., L. Tanigoski, and J. G. Mudd. 1971. Incorporation of ^{14}C-amino acids by malaria (*Plasmodium lophurae*). II. Migration and incorporation of amino acids. Int. J. Biochem. 2:27-40.
30. Suzuki, K. 1965. The pattern of brain gangliosides. II. J. Neurochem. 12:629.
31. Tenforde, T. 1971. Microelectrophoretic studies on the surface chemistry of erythrocytes. *In* J. H. Lawrence and J. W. Gofman (ed.), Advances in biological and medical physics. Academic Press Inc., New York.
32. Trager, W. 1968. Cultivation and nutritional requirements, p. 149-174. *In* D. Weinman and M. Ristic (ed.), Infectious blood diseases of man and animals. Academic Press Inc., New York.
33. Weiss, L., R. Zeigel., O. S. Jung, and I. D. J. Bross. 1972. Binding of positively charged particles to glutaraldehyde-fixed human erythrocytes. Exp. Cell Res. 70:57.

B. STUDIES ON ANTIGENS OF *P. BERGHEI*

I. Protein content of soluble and insoluble fractions of freeze-thawed parasites

Parasites were harvested by our regular sonication harvest method and the parasites were washed once in Alsever's Solution. The parasites were counted on a hemocytometer and adjusted to a predetermined number. The free parasites were freeze-thawed three times in a dry ice-acetone bath and 37°C water bath. The freeze-thawed parasites were spun at 30,000 g for 30 minutes. The pellet was resuspended in the same volume of fluid as was the original suspension, thus making the soluble and insoluble components of the parasites comparable.

Lowry Protein assays were used to determine the protein contained in the samples. Protein determinations were also done on samples of unfractionated freeze-thawed parasites to determine total protein in the samples. Bovine Serum Albumin was used for the standards.

The results of four different runs indicate that about one-third of the protein was released by our normal freeze-thawing procedure (Table 1). There was some degree of variability in the total protein in various preparations which were supposed to contain the same number of parasites. This inconsistency was probably due to fluctuations in the criteria of what was or was not counted as a parasite while counting with the hemocytometer.

The next set of experiments was to determine the optimal number of freeze-thawings needed for a maximum release of soluble protein. The parasites were harvested as before and resuspended to the desired concentration after counting with a hemocytometer. The parasites were distributed in 1 ml aliquots in test tubes. The samples were treated with from 0 to 7 cycles of freeze-thawing, and after centrifugation the Lowry Protein Assay was used to determine the amount of protein in soluble and insoluble fractions.

The percent protein solubilized by one cycle of freeze-thawing was comparable to that released by the maximum numbers of freeze-thawings that were used. Between no freeze thawing and one cycle of freeze-thawing, the percent soluble material increased from about 3% to about 20%. The percent soluble protein remained at about this level for as many cycles of freeze-thawing as we carried out in the experiment (Table 2). We concluded that three cycles of freeze-thawing solubilizes the maximum amount of protein from the parasites which can be obtained from the parasites by this procedure.

Table 1. Protein content (ug/ml) of soluble vs insoluble components of freeze-thawed parasites.

NUMBER OF PARASITES USED	SOLUBLE	INSOLUBLE	TOTAL
5×10^8	400 33%	1120 68%	1240
5×10^8	412 36%	824 72%	1144
2×10^9	2536 39%	3584 57%	6240
2×10^9	1516 31%	3280 68%	4800

Table 2. Effect of various numbers of freeze-thawing cycles on the amount of protein solubilized.

NUMBER OF TIMES FREEZE-THAWED	SOLUBLE	INSOLUBLE
0	20 (3%)	720 (97%)
1	106 (17%)	514 (83%)
2	145 (22%)	500 (78%)
3	128 (20%)	500 (80%)
4	133 (21%)	512 (79%)
5	133 (18%)	610 (82%)
6	151 (22%)	522 (78%)
7	148 (21%)	566 (79%)

II. Cryo-impacting as a technique for solubilizing free parasite protein

In order to fractionate the parasites so that it will be possible to identify stage specific and protective antigens, it is first necessary to convert the parasites to a soluble form. Freeze-thawing as was shown in the previous section (B-I) of this report will only release from 1/5 to 1/3 of the parasite material leaving much membranous material insoluble and thus refractory to analysis. In an attempt to increase the amount of protein that could be solubilized, we subjected the free parasites to the process of Cryo-Impacting (Smucker, R. A. and Pfister, R. M., 1975, Liquid nitrogen Cryo-Impacting: A new concept for cell disruption. Applied Microbiol. 30: 445-449). This procedure has been shown to be effective in disrupting of bacterial cells and even the endospores of *B. megaterium*.

The parasites were harvested and frozen in liquid nitrogen by dropping the parasite suspension directly into the liquid nitrogen filled chamber of the Cryo-Impacting apparatus. We placed about 1 ml of the free parasite suspension containing 5×10^8 cells/ml in the chamber and removed samples after 15, 30, 60, 120, and 150 seconds of Cryo-Impacting. After each period of Cryo-Impacting, a small amount of the frozen powder was removed from the impacting chamber with a spatula, placed in a petri dish and allowed to melt. About 0.1 ml of this liquid was then mixed with 0.9 ml of buffer. These diluted samples were then tested for total protein content, then centrifuged at 30000 g for 30 minutes. The soluble and insoluble fractions were then tested for protein content. We have not yet resolved all of the technical problems of handling the Cryo-Impacted parasites. On melting, the impacted parasites yield a viscous fluid which contains numerous small air bubbles and apparently some small fragments of ruptured parasites.

Although the technical difficulties in pipetting this viscous fluid made it difficult to obtain quantitative results from the time sequence study, it was apparent that even after the shortest period of impacting the product contained very little intact parasite material visible to the naked eye. Nor did the samples after centrifugation yield a visible pellet. The total protein content in each sample was approximately the same as the protein content in the soluble fraction. These observations indicate that Cryo-Impacting disrupts the parasites far more than does freeze-thawing and indicates that this technique may be useful for preparation of parasites for disc gel electrophoresis and other fractionation procedures.

III. Density gradient centrifugation as a technique for separating parasitized erythrocytes and free parasites by stage of development

Plasmodium berghei produces an unsynchronized blood stage infection. Sonic Oscillation followed by differential centrifugation (Prior and Kreier, 1972; 1972a) has been used to obtain free parasites from such infected blood. For the purposes of studies of stage specific antigens, it would be desirable to free the parasites from blood cells all of which contained parasites in the same stage of development or to be able to separate the freed parasites by stage of development. No studies of the density of free blood stage parasites have been reported, but several recent reports of studies designed to separate erythrocytes by stage of development of the contained parasite have appeared.

Lunde and Powers (1976) in attempts to prepare malaria hemagglutination antigens isolated schizonts by use of ficoll gradients. They found that most of the schizonts accumulated in the 20% and in the interface between the 20 and 25% ficoll bands. They collected the schizonts by centrifuging the gradients at 10,000 $\times$ g for 30 minutes.

McAlister and Gordon (1976) obtained schizonts from infected mouse blood using food grade stractan 11. In their experiments, most of the schizonts accumulated in the bands having a specific gravity of 1.043. This layer also accumulated a substantial population of trophozoite infected cells.

Since Plasmodium berghei does not exhibit any substantial degree of synchrony as do the Simian and human malarias, the techniques which will produce highly enriched populations of schizonts and trophozoites with these malarias are not entirely suitable for P. berghei and new techniques must be developed to collect parasites of specific classes from an otherwise heterogenous population of P. berghei parasites.

MATERIALS AND METHODS

Parasite Preparation.

Plasmodium berghei (Walter Reed strain) was maintained in albino Swiss mice. Rats used for these tests were of any breed and weighed between 250 and 500 grams. Each rat was pre-treated with three injections of phenylhydrazine hydrochloride (30 mg/kg) at 48 hour intervals to induce reticulocytosis. The rats were infected by intra-cardial or tail vein injections of parasitized fresh mouse blood 48 hours after the third injection of phenylhydrazine hydrochloride. When the parasitemia reached 50% or more the animal was exsanguinated. The blood was collected by cardiac puncture using a syringe pre-loaded with Alsever's solution (pH 7.2). The blood was centrifuged in a Sorvall RC-2 Automatic

Refrigerated Centrifuge at 2000 RPM for 10 minutes. The supernatant was aspirated and discarded. The blood was washed twice in 0.153M sodium chloride, filtered through glass wool and shredded filter paper to remove leucocytes then washed once more in 0.153M saline. The washed blood was resuspended in saline and was layered on the surface of a discontinuous ficoll gradient. Unless otherwise indicated, all work was done in the cold.

Ficoll Preparation.

A 50% stock ficoll (w/v) solution was made in double distilled water. The ficoll was allowed to dissolve at room temperature. Solutions containing the desired percentages of ficoll were prepared by mixing the appropriate volumes of stock ficoll and 0.153M saline. The gradients were prepared by dispensing 4.0 ml aliquots of ficoll delivered from a 5.0 ml pipet beginning with the highest density. The ficoll was gently run down the side of a thin-wall polyallomer round bottom tube (28.6 x 103.7 mm). The gradients were chilled in an ice bath prior to the layering on the blood.

Centrifugation and Collection of the Bands.

The ficoll gradients were centrifuged in an RC2 refrigerated centrifuge with a swinging bucket head at 5000 RPM for 70, 80, 90, 100, 110, and 120 minutes or at 7000 RPM for 30 and 45 minutes. Afterwards, the bands were collected with a Pasteur pipet. The collected bands were resuspended to a final volume of 15 to 20 ml in 0.153M saline and centrifuged at 5000 RPM for 20 minutes. The supernatant was aspirated and discarded and the remaining pellet was resuspended in 2.5% egg albumin and thin smears were made.

Counting Procedure.

The cells were divided into two categories: mature red blood cells and reticulocytes. Each category was further subdivdided into three classes: uninfected cells, cells containing trophozoites and cells containing schizonts. Parasites containing two or more nuclei were classified as schizonts and multiply parasitized cells were classed by the most advanced stage present. The distribution of parasitized erythrocytes in each band was determined by examining 250 cells on a Giemsa stained thin blood film.

RESULTS

Red blood cells and reticulocytes have different density characteristics. Reticulocytes band out in lighter density bands than the mature red blood cells. In general, our study (Table 1) confirmed that erythrocytes containing parasites were less dense than uninfected cells. The reticulocytes containing trophozoites accumulated in the 20 to 22 percent

Table 1. Distribution (in %) of parasitized erythrocytes before and after centrifugation on a stepwise ficoll density gradient. The gradients were centrifuged at 5000 RPM for 70, 80, 90, 100, 110 or 120 minutes (RET., reticulocytes; RBC, red blood cells; C, uninfected cells; T, cells containing trophozoites; S, cells containing schizonts; Pe., pellet).

		RET.			RBC		
Time (min)	% ficoll	C	T	S	C	T	S
0 (control)		7.4	35.2	13.6	23.4	17.4	3.0
70	20	1.6	52.0	39.0	1.2	4.4	1.8
	21	8.4	56.0	26.4	2.4	4.4	2.6
	22	15.4	45.0	5.6	7.8	24.6	1.6
	23	13.8	14.8	4.8	33.4	30.6	2.4
	24	8.2	5.4	2.2	68.4	14.8	1.0
	Pe.	5.6	3.8	0.8	84.8	4.6	0.4
80	20	0.4	57.2	41.4	0.4	0.0	0.6
	21	13.8	55.2	15.2	1.4	11.2	3.2
	22	25.6	43.4	6.2	8.4	14.6	1.8
	23	15.7	20.0	5.6	16.8	36.0	6.2
	24	6.2	1.3	1.8	75.4	13.8	1.0
	Pe.	6.8	2.2	0.4	78.0	12.6	0.0
90	20	5.4	74.0	15.6	2.0	3.2	0.2
	21	17.8	48.0	7.8	3.4	20.6	2.8
	22	34.6	24.6	4.2	10.6	23.6	3.2
	23	17.6	1.6	00.2	60.6	19.6	0.4
	24	8.4	2.6	0.8	79.4	10.0	0.8
	Pe.	6.6	1.6	0.2	83.4	7.8	0.6
100	20	4.2	65.4	26.2	1.2	1.0	2.0
	21	13.2	73.8	4.6	0.8	6.8	2.0
	22	17.9	47.2	4.2	5.8	23.8	1.0
	23	32.6	20.6	2.0	26.8	17.4	0.6
	24	8.0	2.8	0.4	77.0	10.6	0.2
	Pe.	3.4	1.0	0.4	90.0	5.4	0.0
110	20	4.0	77.2	16.4	1.8	0.8	0.0
	21	9.0	66.6	8.8	4.8	8.0	2.8
	22	20.2	48.4	3.6	10.8	16.2	0.4
	23	21.8	19.2	1.6	27.4	28.6	1.4
	24	19.8	21.0	0.0	36.0	18.4	0.6
	Pe.	7.4	2.0	0.0	88.8	1.6	0.0
120	20	9.0	56.8	22.0	6.0	5.8	0.4
	21	8.0	49.0	8.0	12.0	6.0	3.0
	22	50.2	25.2	0.0	7.2	18.0	0.0
	23	28.0	10.8	0.6	33.6	26.2	0.8
	24	17.0	10.0	1.0	61.0	11.0	0.0
	Pe.	12.6	2.4	0.8	79.4	6.8	0.0

layers while the uninfected reticulocytes were more likely to accumulate in the 22% to 23% layers. Most of the trophozoite-containing erythrocytes accumulated in the 22% to 24% ficoll layers, while the uninfected mature RBC's were recovered in the bands with a density greater than 24% ficoll. Most of the schizont containing cells whether reticulocytes or mature red cells accumulated in the 20 and 21% ficoll layers. It appears that the centrifugation was to equilibrium at 70 minutes in our study as increasing time of centrifugation did not cause much change in the distribution of the cell classes in the bands.

In a second study (Table 2) done with a greater gravity force, applied for shorter times and using a gradient ranging from 14 to 22% ficoll instead of 20% to 24% ficoll, equilibrium was not achieved in 30 minutes but was approached in 45 minutes. The band with the highest proportion of schizonts (i.e., over 80%) was that in the 18% ficoll layer. A population of trophozoite containing reticulocytes accumulated in the 16% ficoll layer. The bulk of these trophozoite containing reticulocytes were ones with multiple infections of large trophozoites. Parasites which were free of host cells accumulated mainly in the 14% ficoll layer, although some were present in the 16 and 18% layers also.

DISCUSSION

There are a variety of variables which must be considered in the separation of erythrocytes based on their buoyant densities. There is a range of densities among the uninfected erythrocytes which is related to the stage of maturation and hence age. At the extreme range of these variations are mature erythrocytes and reticulocytes, which have very different density characteristics. Slight differences in the osmotic pressure of the ficoll bands may cause the cell or the parasite (if the cell is infected) to imbibe or lose water thus altering the cell's true buoyant density. The effect of the parasites on the infected cells is super-imposed on these variables. All of these variables interacting account for the separation obtained.

Uninfected mature red blood cells are the most dense (greater than the density of 24% ficoll); however, once infected, these cells generally become lighter. The bulk of the mature erythrocytes infected with very small trophozoites have densities greater than 23% ficoll indicating that the small parasites have little effect on the density of the host cell. Schizont infected mature erythrocytes, in general, are less dense than uninfected mature erythrocytes and have density characteristics similar to uninfected reticulocytes. The schizont-infected erythrocytes float in 20 to 23 percent ficoll bands.

Uninfected reticulocyte float in the 22% ficoll range, but when infected with large trophozoites become much lighter and float in the 20% layer. Schizont infected reticulocytes have densities which cause them

Table 2. Distribution (%) of parasitized erythrocytes before and after centrifugation on a stepwise ficoll density gradient. The gradients were centrifuged at 7000 RPM for 30 or 45 minutes (RET., reticulocytes; RBC, red blood cells; C, uninfected cells; T, cells containing trophozoites; S, cells containing schizonts; Pe., pellet).

		RET.			RBC		
Time (min)	% ficoll	C	T	S	C	T	S
0 (control)	-	1.0	12.6	5.0	76.4	4.6	0.4
30	14*	-	-	-	-	-	-
	16	13.4	31.8**	19.5	30.5	4.9**	0.0
	18	2.0	12.0	82.7	27.0	1.3	0.0
	20	0.4	48.0	55.2	0.0	4.0	0.4
	22	9.2	65.2	22.8	2.0	1.3	9.2
	Pe.	1.3	3.2	2.0	89.6	4.0	0.4
45	14*	-	-	-	-	-	-
	16	8.0	45.0**	28.0	2.0	15.0**	3.0
	18	1.2	12.0	86.8	0.0	0.0	0.0
	20	0.7	41.5	57.0	0.4	0.4	0.0
	22	8.4	45.2	26.4	1.2	16.0	2.8
	Pe.	0.6	2.2	7.2	88.6	5.4	0.2

*Free parasites and erythrocyte membrane fragments were prominent in the band in this layer.

**These were largely reticulocytes with multiple large trophozoite infections.

to float in the 18 to 20% ficoll bands. In heavily infected blood, some reticulocytes with multiple large trophozoite infections also are of low density.

Reticulocytes with multiple large trophozoite infections float in the 16% ficoll band whereas the bulk of the schizont infected reticulocytes float at the 18 and 22% ficoll layers. If one is attempting to isolate schizont containing cells by gradient techniques, a simple system in which one collects all cells with densities less than 20 or 22% ficoll (Lunde and Powers, 1976) will yield a product containing many reticulocytes with multiple trophozoite infections. As these are even lighter than schizont containing cells a two step gradient is useful for improving the density of the schizont wall.

As can be seen from Table 1, the maximum schizont enrichment was present in the 20% layer when this was the least dense layer in the gradient. However, when lower ficoll densities were used (Table 2) the purest schizont population was present in the 18% band as the host cells with multiple trophozoite infections were retained in the 16% band. Thus, using lighter ficoll solutions not only allows for considerable enrichment in schizonts, but also less contamination.

Free parasites in the parasitized erythrocyte preparations were retained in the 14% ficoll band although some were in the 16% and 18% bands also. Our preliminary studies thus show that parasites freed by sonic oscillation (Prior and Kreier, 1972, 1972a) have density characteristics which cause them to float in bands with densities somewhere between 14 and 18% ficoll. Thus gradient centrifugation may be useful for collecting free parasites as well as parasitized erythrocytes.

LITERATURE CITED

Lunde, M. N. and Powers, K. G. 1976. The preparation of Malaria Hemagglutination Antigen. Annals of Tropical Medicine and Parasitology 70 (3): 283-219.

McAlister, R. O. and Gordon, D. M. 1976. Schizont-Infected Cell Enrichment in Rodent Malaria. The Journal of Parasitology 62 (5): 664-669.

Prior, R. B. and Kreier, J. P. 1972. Plasmodium berghei Freed from Host Erythrocytes by a Continuous-Flow Ultrasonic System. Experimental Parasitology 32: 239-243.

Prior, R. B. and Kreier, J. P. 1972 a. Isolation of Plasmodium berghei by use of a Continuous-Flow Ultrasonic System: A Morphologic and Immunological Evaluation. Proceedings of the Helminthological Society, Washington, 39: 563-574.

IV. *Plasmodium berghei*: Immunization of rats with antigens from a population of free parasites rich in merozoites. (manuscript)

Plasmodium berghei: Immunization of rats with antigens from a population of free parasites rich in merozoites.*

K. W. Saul and J. P. Kreier

1. Research associate (K.W.S.) and Professor (J.P.K.) in the Department of Microbiology, The Ohio State University, Columbus, Ohio 43210

* This research was supported in part by contract number DADA 17-72-C-2072 from the U.S. Army Medical Research and Development Command, and is contribution # to the Army Research Program on Malaria.

Abstract

A population of P. berghei rich in merozoites was obtained by differential centrifugation of parasites freed from infected red cells by continuous flow sonication. These parasites were used as immunogens in vaccination studies. Rats of three age groups were used to evaluate the vaccine with or without the addition of adjuvants.

Immunization with the freeze-thawed free parasites without adjuvants caused enhancement of infections in young weanling rats. In contrast, in young adult rats and mature adult rats, the antigen without adjuvants had a slight protective effect. Immunization with freeze-thawed free parasites with adjuvant gave protection in rats of all weight groups. Five x 10^8 freeze-thawed free parasites with saponin given intramuscularly in a single dose protected 70 to 90 gram CDF rats from the effects of challenge one week later and this protection persisted for 8 weeks, the longest time tested. The freed parasites did not lose antigenicity when stored frozen for two weeks. Saponin was at least as effective an adjuvant as Freund's Complete Adjuvant (FCA) with this antigen. The soluble portion of the freeze-thawed parasite preparation appeared to be as protective as the insoluble portion and injection of material washed from the surfaces of the parasites gave about the same degree of protection as did injections of preparations of freeze-thawed parasites. Rats which were given freshly prepared whole unbroken free parasites emulsified in FCA developed severe infections on challenge one week later and if unchallenged sometimes developed parasitaemias after 9 or 10 days.

Introduction

With the growing awareness of the limitations of the conventional methods of malaria control there has developed a new interest in malaria immunology (W.H.O., 1975). The recent remarkable advances in immunological knowledge and technology have greatly facilitated and encouraged study of this particular aspect of malaria.

Perhaps the salient feature that is emerging from current research is the recognition of the complexity of the immune response to plasmodial infection. The need today is to define the components of the immune response, to assess the respective contributions of the cellular and humoral mechanisms to the control of the parasite and to characterize and purify the specific antigens important for protection. Some antigens, possibly only a few, may be particularly important in initiating protective immunity. Many probably have no such function, while others may initiate responses that are frankly harmful to the host.

Attempts at immunization of birds or monkeys by injection of killed or attenuated blood forms or sporozoites obtained from infected mosquitos go back to the first quarter of the century (Sergent and Sergent, 1910). Research on immunization against malaria has been stimulated by early reports of success in immunization against malaria of monkeys, birds, and rats (Mulligan and Sinton 1933; Freund et al., 1945, 1945a, 1948; Corradetti et al., 1966). More recently various methods of vaccination employing fractions of plasmodial parasites from the blood of rodents (Simpson, et al., 1974 ~~1974~~; (D'antonio, 1972) and monkeys (D'antonio, 1974) or vaccination with irradiated sporozoites (Nussezweig et al., 1967), formalized gametocytes

(Gwadz 1976), exoerythrocytic merozoites (Holbrook et al., 1974) or erythrocytic merozoites (Mitchell et al., 1975) have further encouraged workers to search for practical vaccines against malaria.

Sonic oscillation carried out in a continuous flow sonication system releases intraerythrocytic P. berghei. The sonicated preparations when subjected to differential centrifugation, yield populations of P. berghei rich in merozoites (Prior and Kreier 1972; 1972a; Prior et al., 1973; Kreier et al., 1976). The parasites obtained by these procedures are morphologically intact, and antigenic. Other workers have also obtained merozoites (Mitchell, Butcher, and Cohen, 1973; Dennis et al., 1975) and used them to study immunization against plasmodial infection (Mitchell et al., 1975).

Since it appears that immunity to plasmodia can be at least partially explained in terms of antibody action against merozoites, (Cohen and Butcher 1970; Cohen et al., 1961; 1969; 1972; Jerusalem et al., 1971; Hamburger and Kreier 1975, 1976), it is, therefore, very likely that protection from the ravages of the blood forms of plasmodial parasites will be conferred by inoculation with antigens from erythrocytic merozoites.

This study provides data on the immunization of rats against Plasmodium berghei with antigens of a sonically freed preparation of erythrocytic parasites rich in merozoites.

MATERIALS AND METHODS

Parasites

The *Plasmodium berghei* strain used in these studies was obtained from the Walter Reed Army Institute of Research. It is highly pathogenic to mice and causes death, generally within a week after onset of patency. Weanling rats may die when infected with this strain but young adult and adult rats usually survive.

The parasites were grown in adult CDF rats infected by the intravenous route with fresh parasitized rat blood. To reduce the possibility of antigenic variation when populations of parasites were being produced, parasites of the reference strain stored in Alsever's solution plus 10% (v/v) glycerol in liquid nitrogen (-193 C.) were used to initiate infections in the donor rat each time parasites were produced for antigen or challenge.

Animals

Male CDF rats (Fisher 344, Charles River Animal Laboratories, N. Wilmington, Massachusetts) were used throughout the experiments for both antigen production, as the source of challenge inocula, and as the test animals. The rats used were either young weanling rats weighing 40-50 grams, young adult rats weighing 70-90 grams, or mature adult rats weighing 250-280 grams.

The test animals were alloted by a modified random selection system into the various groups. The rats were weighed before allotment, grouped by weight and were randomly alloted to the test groups from the groups of equal weight. This was done to assure that the average and range of weights in all groups were nearly equal.

Antigens

Harvesting of free parasites (FP): Free parasites were harvested from the blood of infected rats. The blood was collected from the animals after parasitaemia had reached about 50%. In order to obtain 50% parasitaemia in the harvested blood, it was necessary to increase the reticulocyte count in the rats. To do this, on day 0 the rats received intraperitoneally 30 mg/kg of phenylhydrazine hydrochloride in a 1.5% aqueous solution (Kreier _et al._, 1976). This dosage was repeated on day 2. The animals were infected by intravenous injection of one ml. of heavily infected rat blood on day 5. Blood was harvested 3 days later. Infected blood was collected into Alsever's solution and washed twice by centrifugation at 650 g for 10 minutes. A 10% suspension of the infected blood cells was passed through a column of filter paper powder prepared in 50 ml. syringes to remove leukocytes. After an additional washing, the blood cells were diluted to give a 10% suspension. The erythrocytes were disrupted by treatment in a continuous flow sonication system by methods previously described (Prior and Kreier, 1972, 1972a). The suspension of sonicated blood cells was placed in 10 cm. high tubes (8 cm. fluid column) and was centrifuged at 650 g for 10 minutes and the supernatant which contained predominantly small parasites was collected and washed in the same way once in Alsever's solution by centrifugation at 2520 g for 10 minutes. While these centrifugation conditions differ somewhat from those previously reported (Hamburger and Kreier, 1975; Kreier _et al._, 1976), they provide a larger parasite yield than those previously reported and for the purposes of immunization the increased amount of contamination by host material is not significant.

Free parasites were used fresh or after storage in liquid nitrogen in the whole unbroken state. Freeze-thawed antigens were prepared just before use by three cycles of freezing in a dry ice bath and thawing in tepid water.

Soluble antigens were separated from insoluble ones by centrifugation of three times freeze-thawed parasites at 30,000 g's for 30 minutes. The dose of the fractions given was the amount of the fraction obtained from the comparable dose of unfractionated parasites (i.e. 5×10^8 free parasites).

For comparisons of immunogenicity of whole unbroken parasites with solubilized parasites, fresh free parasites or freeze-thawed preparations were emulsified with FCA as described by Mitchell et al., (1975). Infectivity of these preparations were tested by intramuscular injection into mice and rats.

The wash off antigen was prepared by suspending about 5×10^9 whole free parasites in 2 ml. cold physiological saline. The free parasites were spun gently with a magnetic stir bar and plate in the cold for 2 hours. The washed free parasites were then separated from the released material by centrifuging at 30,000 g for 30 minutes. Saponin was added to the antigen as an adjuvant. Each 0.2 ml. of the supernatant contained one dose of antigen.

Disc gel electrophoresis analysis was carried out on the capsular antigen by methods previously used (Kreier et al., 1976). Relative mobilities were calculated and these were compared to those of a freeze-thawed extract of small parasites freed by sonic treatment.

Erythrocyte membrane antigen used as control antigen, was made by water lysis of CDF rat red cells. The dose used was the volume of membranes equal to the volume of 5×10^8 free parasites.

Immunization

A number of vaccination experiments were carried out, these experiments were designed to determine the relationship of a variety of factors to the ability of rats to respond to immunization with antigens of small free erythrocytic forms of *Plasmodium berghei*. These variables included the maturity of the rats (indicated by the rats weight), the type and dose of antigen, the presence or absence of adjuvants and the type of adjuvant, the number of doses of antigen given and the time between immunization and challenge (Table I). All vaccines were given intramuscularly.

Results

<u>Relationship of antigen dose to immunological response in weanling (40-50 g.) and young adult (70-90 g.) rats:</u>

In weanling rats (40-50 g.) given various doses of freeze-thawed *P. berghei* parasites without adjuvant of any kind the severity of the infection following challenge was roughly proportional to the dose (Figure 1A). In rats of this age group the injection of plasmodial antigen without adjuvant enhanced the severity of the infection. When saponin was added to the antigen the infections were generally milder in the animals which received plasmodial antigen than in those which received red cell membranes and this amelioration was in general proportional to the dose (Figure 1B). The infections in the 40 to 50 gram rats which received red cell membrane antigen with saponin (Figure 1B) were more severe than were the infections in the 40-50 g. rats which received red cell membrane antigen without saponin (Figure 1A).

The infections following challenge in the young adult rats (70-90 g.) which had been given various doses of free parasite antigen without adjuvant were in general more severe than were the infections in the respective red cell membrane control rats but there was no obvious relationship between amount of antigen given and the degree of enhancement of the infection (Figure 1C). The young adult rats which received various doses of free parasite antigen with saponin added at first developed infections which were similar or possibly slightly more severe than those in the rats which received red cell membranes with saponin but they aborted their infections more rapidly than did the rats which received red cell membranes and controlled their relapses better. The degree of amelioration of the relapses in the rats of the various groups was roughly proportional to the size of the dose of antigens the rats received (Figure 1D).

In the young adult animals, the infections after challenge in the rats which received red cell membranes with saponin added (Figure 1D) were not or possibly only slightly more severe than were the infections in the young adult rats which received red cell membranes without saponin (Figure 1C).

The relationship between parasitaemia following challenge and time after immunization at which challenge takes place.

The pattern of parasitaemia following challenge of 70-90 gram rats which had been given one injection of antigen from 5×10^8 free parasites without any adjuvant was not very different from the pattern of parasitaemia in similar rats given an equivalent volume of red cell membranes. This was true whether the challenge was at one, two, four or eight weeks after immunization (Figures 2E, F, G and H). In rats which received the plasmodial antigen with saponin added however, the parasitaemias following challenge were much milder than they were in the respective control rats which received red cell membranes. The least ambiguous differences occurred in rats challenged at one and two weeks following immunization but fairly clear differences occurred at 4 and 8 weeks also (Figures 2A, B, C and D).

Comparison of the immunogenicity of freshly prepared free parasite antigen and free parasite antigen which had been stored frozen for two weeks.

Storage in the frozen state had no obvious effect on the antigen, as parasitaemias following challenge were almost identical in the rats which received the stored antigen and those which received the fresh antigen (Figure 3).

Effect of maturity of the rats upon immunization.

In young weanling rats (40-50 g.) four injections of 5×10^8 freeze-thawed free parasites without adjuvant markedly aggravated the parasitaemia following challenge (Figure 4A). In young (70-90 g.) adult rats (Figure 4B) and in mature (250-280 g.) adult rats (Figure 4C) identical treatment reduced the severity of the secondary relapses which occurred in the rats which received erythrocyte membrane antigen but otherwise had little effect. In rats of the same age groups which received the same doses of antigen on the same schedule but with saponin added (Figures 4D, E, F) the parasitaemias following challenge were lower than they were in the respective control rats. In the weanling (40-50 g.) and young adult (70-90 g.) rats (Figures 4D and E) which received the P. berghei antigen with saponin the primary parasitaemias developed almost as they did in the unvaccinated controls but the vaccinated animals brought the parasitaemias under control more rapidly than did the control animals. The mature adult (250-280 g.) rats which received plasmodial antigen in saponin had mild primary parasitaemias and no relapses (Figure 4F).

Comparison of the relative effects of saponin and Freund's complete adjuvant on the immune response.

Young adult rats (70-90 g.) which received 4 injections of 5×10^8 freeze-thawed parasites with saponin (Figure 5A) had about the same degree of amelioration of their parasitaemias as did young adult rats which received the antigen in FCA (Figure 5B).

Comparison of the relative immunogenicity of the soluble and insoluble components of freeze-thawed parasites.

Young adult rats (70-90 g.) which received one injection containing the insoluble components of 5×10^8 freeze-thawed free parasites with saponin developed parasitaemias after challenge which were very similar to the parasitaemias which developed after challenge in similar rats which received all of the components of 5×10^8 freeze-thawed free parasites. The parasitaemias after challenge in the rats which received only the soluble components were slightly more persistent than were the parasitaemias in the other two groups of immunized rats, while all of the immunized rats had milder parasitaemias after challenge than did the rats which received red cell membranes (Figure 6).

Comparison of the relative immunogenicity of whole unbroken and freeze-thawed free parasites.

Fresh unbroken and freeze-thawed free parasites to which saponin was added were essentially equivalent in their immunogenicity as parasitaemias after challenge were similar in rats which received a single dose of 5×10^8 parasites prepared either way. Both preparations stimulated a substantial protective response in the injected rats, for the parasitaemias in the rats which received the antigen were considerably lower than those in the control rats which received red cell membranes (Figure 7A). The rats which received 5×10^8 freeze-thawed free parasites emulsified in FCA also developed considerable immunity as their parasitaemias were similar to those in the rats which received parasites in saponin and were considerably less severe than the parasitaemias in the rats which received erythrocyte membranes

in FCA. Parasitaemias in the rats which were given a single dose of fresh whole unbroken parasites in FCA and challenged one week later were more severe than were parasitaemias in the rats of the red cell control group. Mice and rats given fresh whole parasites in FCA sometimes became infected between one and two weeks later. Infection did not occur in mice or rats given freeze-thawed antigen in FCA or with antigen of any type prepared with saponin.

Comparison of the immunogenicity of an antigen washed from the surface of free parasites to that of the unfractionated parasite antigen.

On disc gel analysis (Figure 8, right hand side), the preparation obtained by washing small free parasites contained one major band the components of which had a relative mobility of between 56 and 60 (Table II). Minor bands corresponding to the bands present in the soluble fractions of freeze-thawed free parasites were also present.

The unfractionated freeze-thawed preparation had only minor bands in the 56 to 60 region (Figure 8, left hand side and Table II). The 70-90 gram rats which were given one injection of the amount of material washed from 5×10^8 free parasites containing saponin developed parasitaemias after challenge one week later not very different from the parasitaemias in the rats which were given 5×10^8 freeze-thawed free parasites with saponin. Both these groups of rats had parasitaemias after challenge which were much less severe than were the parasitaemias in the rats which received red cell membrane control antigen (Figure 9).

Discussion

The selection of a suitable laboratory animal for testing the immunogenicity of P. berghei vaccines may determine the success of the study. The mouse does not seem to have the immunological and physiological ability to control P. berghei (Krettle and Nussenzweig 1974) and therefore probably cannot respond well to plasmodial vaccines either. The young adult CDF rat (70-90 g.) which like man can usually control plasmodial infection has been demonstrated in this study to be a good laboratory animal in which to evaluate plasmodial antigens. The infection in these animals is sufficiently intense that parasitaemias can be used as a measure of the success of immunization, and their immune response is sufficient to elicit an observable level of protection when appropriately stimulated. It is also possible to study the development of the immune response in these rats as their ability to control their infections increases as they mature.

When the freeze-thawed Plasmodium berghei parasite preparation was used as a vaccine without adjuvants there was a demonstratable enhancement of the infection in 40-50 gram rats. In contrast, in 70-90 and 250-280 gram rats, the antigen without addition of adjuvant had a demonstratable protective effect although it was slight in the 70-90 gram rats. A study by Gravely, et al., (1976) indicated that during infection of weanling CDF rats (40-50 g.) there is a reduction in numbers of T and B cells and that the animal does not have the capacity to mount a successful immunological response to the infection and dies of an overwhelming parasitaemia. They also showed that during infection of adult CDF rats (70-90 g.) the T and B cells proliferate, the rat develops a successful immunological response, and eventually is resistant

to the disease. Thus injection of plasmodial antigen into rats causes immunological responses which would be expected from what is known to occur in the lymphocyte populations of rats which encounter plasmodial antigen as a result of infection.

The transfer of immune splenic T lymphocytes did not protect CDF rats against P. berghei, although transfer of B cells alone afforded protection (Gravely and Kreier, 1976). This indicates the importance of antibody in immunity to plasmodia. Thymus-derived (T) lymphocyte stimulation may be critical in developing immunological protection, but these cells most likely function in a "helper cell" capacity in antibody induction (Brown, 1973; Gravely and Kreier, 1976), and are not needed for the implementation of the developed immunity. It has been demonstrated (Hamburger and Kreier 1975, 1976) that administration of protective antibody affects the duration of the prepatent period and the early stages of the infection but has little effect in the later stages and on the final result of the infection, probably because the administered antibody is rapidly eliminated. None of the immunization procedures we carried out affected the duration of the prepatent periods which indicates that little or no protective antibody was in the serum of the immunized animals at the time of challenge. The differences between the immunized groups and the control groups occurred following challenge and after the primary stage of parasitaemia. This indicates that immunization with free parasites brought about changes in the cellular components of the immune system which probably resulted in the development of memory cells. These memory cells did not start secreting antibody until after they were given an additional stimulus from the challenge infection. It was thus an anamnestic response in the immunized rats which controlled the infection.

The enhancement of infection we observed in 40-50 gram rats given killed parasite antigen without adjuvants indicates that the inability of the young rat to control malaria is a result of the action of parasite antigen on the cells of the young rats immunological system and not just some other physiological characteristic of young rats such as susceptibility of young red cells to parasites as suggested by Zuckerman (1970).

Vaccination of 40-50 gram rats with free parasites without adjuvants resulted in enhancement of infections. When adjuvant was added, on the other hand, the antigen was protective to rats in all weight groups. In rats given the antigen with adjuvant, infections were milder than in controls, and the peak parasitaemias and patent periods were reduced. The adjuvants commonly used in experimental work are the oil and water mixtures such as Freund's adjuvant used for experimental malaria immunization by Freund *et al*., (1945, 1945a, 1948), Thomson *et al*., (1947), Coffin (1951), Brown *et al*., (1970), or adjuvant 65 used by Schenkel *et al*., (1973, 1975). The results of these workers experiments were variable but in general would indicate that plasmodial antigens in FCA or adjuvant 65 stimulate an adequate level of protective immunity. Unfortunately FCA and all other oil based adjuvants are unacceptable for use in humans because of the abscesses and oil embolisms which often result from their use.

When we compared the efficiency of saponin and FCA as adjuvants, we observed that rats which received free parasites with saponin had as good or better immunity as rats which received free parasites incorporated into FCA. Saponin given in the amounts used in this study did not appear to cause abscesses or other persistent effects but did cause local inflammation. It is possible that the cellular infiltration and proliferation associated

with the inflammation which characterizes adjuvant reactions is necessary to produce the desired immunological response. Saponin, used in this study as an adjuvant, has also been reported by other workers to have adjuvant effects (Richards, 1966; Desowitz, 1975). Our results support those of Desowitz, who reported that saponin improves the immunological characteristics of plasmodial antigens.

The 40-50 gram rats which received red cell membranes with saponin developed more severe infections than the 40-50 gram rats which received red cell membranes without saponin. In the 70-90 gram rats this difference did not occur. Saponin injection into the young rats thus increased susceptibility to the infection but the antigen in saponin partially reversed the effect of saponin alone. The amount of saponin given to the small rats caused quite severe but transitory inflammations at the site of injection. If it entered the circulation it could have caused some hemolysis and a compensatory reticulocytosis which would have increased the rats susceptibility to infection. The immune response stimulated by the antigen partially reversed this susceptibility. The larger rats with more body mass were apparently not affected systemically by the saponin.

It has been suggested that the immunogenic effect of malarial antigens may ultimately prove to reside not in a soluble component of the plasmodium but in a relatively insoluble one (Jacobs 1943). In this work we carried out several experiments to test this hypothesis. When the freeze-thawed preparation was separated into soluble and insoluble components by centrifugation, protective ability resided in both components. Freeze-thawing may dislodge some but not all of the antigens from the membranes. If this in fact occurs, then antigens capable of stimulating a protective response

may in fact be membrane associated as suggested by Speer et al., (1976) and still appear in the soluble component of the freeze-thawed parasites. The protection stimulating characteristics of the preparation we obtained by washing the free parasites supports more strongly the proposal that a soluble antigen is important in malarial immunity. Malaria merozoites have been reported to have a relatively loosely attached surface coat (Miller et al., 1975). While we do not yet have any proof that our wash off preparation is in fact surface coat, the disc gel analysis indicates that it is largely, but not entirely, a concentrated preparation of at most a few of the components present in the soluble freeze-thawed parasite preparation. A surface coat or capsular preparation would be the most likely material to be washed from the parasites by the washing procedure we used.

Antigenic variation has been reported to occur in plasmodia and the variable antigen is reported to be associated with soluble antigens, (Brown 1974). The procedures using stabilates for antigen production and challenge which we followed in this study were specifically designed to avoid an effect of antigenic variation upon our test system but will permit the controlled study of antigenic variation if it occurs. Capsular material of trypanosomes appears to be the variable antigen (Vickerman and Luckins 1969; Seed 1972). A surface coat would be an excellent candidate as the seat of a variable antigen on plasmodia also.

We observed that injection of fresh unbroken parasites emulsified in FCA appeared to cause an increase in the severity of infection on challenge of the test rats one week later, but that injection of freeze-thawed parasites under the same conditions gave protection. We also observed that unbroken

fresh parasites emulsified in FCA could cause infection. Cohen and his colleagues (Mitchell et al., 1975) reported that fresh P. knowlesi merozoites in FCA stimulate complete immunity to challenge in rhesus monkeys. Our immunization was with a single dose and challenge was one week later just when some of the animals were developing patent infections from the vaccine. The monkeys which Mitchell et al., (1975) vaccinated with merozoites in FCA were given a series of injections over a fairly prolonged period and could possibly have been preimmunized by the vaccination procedure well before challenge.

Only Cohen and his associates (Mitchell et al., 1975) have reported an anti-plasmodial vaccination procedure which yielded complete protection. In our study, as in most other studies, there has been a reduction in the pathological effects of the disease following vaccination rather than absolute protection. Many have argued that this reduced pathological effect may just possibly be the best available and useful to help combat human malaria (Sinton, 1939; Edozien, 1964; Desowitz et al., 1966). If complete protection is in fact not possible then the administration of plasmodial antigens with an acceptable adjuvant may be justified if it tides the individual over the severe clinical attacks following first exposure while permitting him to obtain treatment or to establish active immunity to the parasite.

References

Brown, K. N., 1973. Thymus-derived (T) lymphocytes in the protective immune response to malaria. 9th International Congress of Tropical Medicine and Malaria. Abst. No. 398, 276.

Brown, K. N., 1974. Antigenic variation and immunity to malaria p. 35-46 in Parasites in the immunized host: mechanisms of survival. Ciba Foundation Symposium 25 (new series) Associated Scientific Publishers. Amsterdam.

Brown, K. A., Brown, I. N., and Hills, L. A., 1970. Immunity to malaria. I. Protection against Plasmodium knowlesi shown by monkeys sensitized with drug-suppressed infections or by dead parasites in Freund's adjuvant. Experimental Parasitology 28, 304-317.

Coffin, G. S., 1951. Active immunization of birds against malaria. Journal of Infectious Diseases 89 1-7.

Cohen, S., and Butcher, G. A., 1970. Properties of protective malarial antibody. Immunology 19, 369-383.

Cohen, S., Butcher, G. A., and Mitchell, G. H., 1972. In vitro studies of malarial antibody. Proceedings Helminthological Society. Washington 39 (special issue), 231-237.

Cohen, S., Butcher, G. A., and Crandall, R. B., 1969. Action of malarial antibody. Nature 233, 368-371.

Cohen, S., McGregor, I., and Carrington, S., 1961. Gamma globulin and acquired immunity to human malaria. Nature 192, 733-737.

Corradetti, A., Verolini, F. and Bucci, A., 1966. Resistenza a Plasmodium berghei da parte di ratti albini precedentemente immunizzati con P. berghei irradiato. Parassitologia 8, 133-145.

D'antonio, L. E., 1972. Plasmodium: A resume of the isolation of a vaccine fraction by the french pressure cell technique. Experimental Parasitology 31, 75-81.

D'antonio, L. E., 1974. Vaccination against malaria: Evaluation of a plasmodial vaccine fraction and a preliminary study of some immunological adjuvants in a primate model system: A brief report. Journal of American Osteopathic Association 39, 649-652.

Dennis, E. D., Mitchell, G. H., Butcher, G. A. and Cohen, S., 1975. In vitro isolation of Plasmodium knowlesi merozoites using polycarbonate sieves. Parasitology 71 475-481.

Desowitz, R. S., 1975. Plasmodium berghei: Immunologic enhancement of antigen by adjuvant addition. Experimental Parasitology 38, 6-13.

Desowitz, R. S., Saave, J. J., and Stein, B., 1966. The application of the indirect hemagglutination test in recent studies on the immunoepidemiology of human malaria and the immune response in experimental malaria. Military Medicine 131, 1157-1166.

Edozien, J. C., 1964. Comments on immunization against malaria. American Journal of Tropical Medicine and Hygiene 13, 233-234.

Freund, J., Sommer, H. E., and Walter, A. W., 1945. Immunization against malaria: Vaccination of ducks with killed parasites incorporated with adjuvants. Science 102, 200-202.

Freund, J., Sommer, H. E., and Walter, A. W., 1948. Immunization of monkeys against malaria by means of killed parasites with adjuvants. American Journal of Tropical Medicine 28, 1-22.

Freund, J., Thomson, K. J., Sommer, H. E., Walter, A. W., and Schenkein, E. L., 1945a. Immunization of Rhesus monkeys against malarial infection (Plasmodium knowlesi) with killed parasites and adjuvants. Science 102, 202-204.

Gravely, S., Hamburger, J., and Kreier, J. P., 1976. T and B cell population changes in young and adult rats infected with Plasmodium berghei. Infection and Immunity 14, 178-183.

Gravely, S. and Kreier, J. P., 1976. Adoptive transfer of immunity to Plasmodium berghei with immune T and B lymphocytes. Infection and Immunity, 14, 184-190.

Gwadz, R. W., 1976. Malaria: Successful immunization against sexual stages of Plasmodium gallinaceum. Science 193, 1150-1151.

Hamburger, J., and Kreier, J. P., 1975. Antibody-mediated elimination of malaria parasites (Plasmodium berghei) in vivo. Infection and Immunity 12, 339-345.

Hamburger, J. and Kreier, J. P., 1976. Plasmodium berghei: The demonstration of protective activity in serum of recovered rats by the use of free blood stage parasites. Experimental Parasitology 40, 158-169.

Holbrook, T. W., Palczak, N. C., and Stauber, L. A., 1974. Immunity to exoerythrocytic forms of malaria. III. Stage specific immunization of turkeys against exoerythrocytic forms of Plasmodium fallax. Journal of Parasitology 60, 348-354.

Jacobs, H. H., 1943. Immunization against malaria. Increased protection by vaccination of ducklings with saline-insoluble residues of Plasmodium lophurae mixed with bacterial toxin. American Journal of Tropical Medicine 23, 597-606.

Jerusalem, C., Weiss, M. L. and Poels, L., 1971. Immunologic enhancement in malaria infection (Plasmodium berghei). The Journal of Immunology 107, 260-268.

Kreier, J. P., Hamburger, J., Seed, T. M., Saul, K., and Green, T., 1976. Plasmodium berghei: Characteristics of a selected population of small free blood stage parasites. Tropenmedizin und Parasitologie 27, 82-88.

Krettle, A., and Nussenzweig, R., 1974. Depletion of T and B lymphocytes during malarial infections. Cell. Immunology, 3, 440-446.

Miller, L., Aikawa, M., and Dvorak, J., 1975. Malaria (Plasmodium knowlesi) merozoites: Immunity and surface coat. Journal of Immunology 114, 1237-1242.

Mitchell, G. H., Butcher, G. A., and Cohen, S., 1973. Isolation of blood stage merozoites from Plasmodium knowlesi malaria. International Journal for Parasitology 3, 443-445.

Mitchell, G. H., Butcher, G. A., and Cohen, S., 1975. Merozoite vaccination against Plasmodium knowlesi malaria. Immunology 29, 397-407.

Mulligan, W. H., and Sinton, J. A., 1933. Studies in immunity to malaria. Part II. Superinfections with various strains of monkey malarial parasites. Records of the Malaria Survey of India 3, 529-568.

Nussenweig, R. S., Vanderberg, J., Most, H., and Orton, C., 1967. Protective immunity produced by injection of X-irradiated sporozoites of Plasmodium berghei. Nature 216, 160-162.

Prior, R., and Kreier, J. P., 1972. Plasmodium berghei freed from host erythrocytes by a continuous flow ultrasonic system. Experimental Parasitology 32, 239-243.

Prior, R., and Kreier, J. P., 1972a. Isolation of Plasmodium berghei by use of a continuous flow ultrasonic system: A morphological and immunological evaluation. Proceedings of the Helminthological Society of Washington 39, 563-574.

Prior, R., Smucker, R. A., Kreier, J. P., and Pfister, R. M., 1973. A comparison by electron microscopy of Plasmodium berghei freed by ammonium chloride lysis to P. berghei freed by ultrasound in a continuous-flow system. Journal of Parasitology 59, 200-201.

Richards, W. H. G., 1966. Active immunization of chicks against Plasmodium gallinaceum by inactivated homologous sporozoites and erythrocytic parasites. Nature 212, 1492-1494.

Schenkel, R. H., Cabrera, E. J., Barr, M. L., and Silverman, P. H., 1975. New adjuvant for use in vaccination against malaria. The Journal of Parasitology 61, 549-550.

Schenkel, R. H., Simpson, G. T., and Silverman, P. H., 1973. Vaccination of Rhesus monkeys (Macaca mulatta) against Plasmodium knowlesi by use of nonviable antigen. Bulletin World Health Organization 48, 597-604.

Seed, J. R., 1972. Trypanosoma gambiense and T. equiperdum: Characterization of variant specific antigens. Experimental Parasitology 31, 98-108.

Sergent, Et., and Sergent, Ed., 1910. Sur l'immunite dans le paludisme des oiseaux. Conservacion in vitro des sporozoites de Plasmodium relictum. Immunite relative obtenue par inoculation de ces sporozoites. Comptes Rendus Academy Science 151, 407-409.

Simpson, G. L., Schenkel, R. H., and Silverman, P. H., 1974. Vaccination of Rhesus monkeys against malaria by use of sucrose density gradient fractions of Plasmodium knowlesi antigens. Nature 247, 304-305.

Sinton, J. A., 1939. Studies in immunity in malaria. IV. The effect of drugs upon the development of immunity and its relationship to the principles of treatment. Journal of the Malaria Institute of India 2, 191-216.

Spear, C. A., Silverman, P. H., and Barr, M. L., 1976. Ultrastructural study of Plasmodium knowlesi antigen used in vaccination of Rhesus monkeys. J. Protozoology, 23, 437-442.

Thomson, K. J., Freund, J., Sommer, H. E., and Walter, A. W., 1947. Immunization of ducks against malaria by means of killed parasites with or without adjuvants. American Journal of Tropical Medicine 27, 79-105.

Vickerman, K. and Luckins, A. G., 1969: Localization of Variable antigens in the surface coat of Trypanosoma brucei using ferritin conjugated antibody. Nature 224, 1125-1126.

Who, 1975. Developments in malaria Immunology. World Health Organization Technical Report (series 579). World Health Organization. Geneva, Switzerland.

Zuckerman, A., 1970. Malaria of lower animals p. 793-829, in G. J. Jackson, R. Herman, and I. Singer (eds) Immunity to Parasitic Animals Vol 2. Appelton-Century-Crofts, New York.

TABLE I: Designs of Vaccination Experiments - showing variables in each experiment.

Fig.#	Rats in group	# of groups	Rat weight at start of experiment (grams)	Antigen type	Control antigen	Adjuvant type	Dose size (# of parasites)	# Doses 1 week intervals	Time to challenge
1) The relationship of antigen dose to immunological response in weanling (40-50g) and young adult (70-90g) rats.									
A	5	6	40-50	F-T	RCM	none	$1x10^9$ $5x10^7$ $5x10^8$ $2.5x10^8$ $2.5x10^7$	4	2
B	3	6	40-50	F-T	RCM	saponin	$1x10^9$ $5x10^8$ $2.5x10^8$ $5x10^7$ $2.5x10^7$	4	2
C	5	6	70-90	F-T	RCM	none	$1x10^9$ $5x10^7$ $5x10^8$ $2.5x10^8$ $2.5x10^7$	4 4	2 2
D	4	6	70-90	F-T	RCM	saponin	$1x10^9$ $5x10^8$ $2.5x10^8$ $5x10^7$ $2.5x10^7$	4	2
2) Determination of optimum time after immunization for challenge.									
A	5	8	70-90	F-T	RCM	none	$5x10^8$	1	1,2,4,8
B	4	8	70-90	F-T	RCM	saponin	$5x10^8$	1	1,2,4,8
3) Effect of storage for 2 weeks at -20 C on antigenicity.									
A	7	3	250-280	F-T Fr SF	RCM	none	$5x10^8$	4	2
4) Effect of rats' maturity upon immunization.									
A	5	2	40-50	F-T	RCM	none	$5x10^8$	4	2
B	10	2	70-90	F-T	RCM	none	$5x10^8$	4	2
C	7	2	250-280	F-T	RCM	none	$5x10^8$	4	2
D	3	2	40-50	F-T	RCM	saponin	$5x10^8$	4	2
E	5	2	70-90	F-T	RCM	saponin[2]	$5x10^8$	4	2
F	4	2	250-280	F-T	RCM	saponin	$5x10^8$	4	2
5) Comparison of effects of saponin and Freund's complete adjuvant on antigenicity.									
A	4	2	70-90	F-T	RCM	saponin	$5x10^8$	4	2
B	5	2	70-90	F-T	RCM	FCA[1]	$5x10^8$	4	2
6) Comparison of relative antigenicity of soluble and insoluble components of the parasites.									
A	4	4	70-90	F-T, I or S	RCM	saponin	$5x10^8$	1	1
7) Comparison of relative immunogenicity of whole unbroken and freeze-thawed parasites.									
A	4	3	70-90	F-T or UnB	RCM	saponin	$5x10^8$	1	1
B	4	3	70-90	F-T or UnB	RCM	FCA	$5x10^8$	1	1
9) Comparison of the relative antigenicity of unfractionated antigen to the antigenicity of the surface coat.									
[illegible]	4	3	70-90	F-T-W or SC	RCM	saponin	$5x10^8$	1	1

Table I

Explanation of Symbols

F-T	Freeze-thawed free parasites
RCM	Red cell membranes
Fr	Freshly prepared free parasites
S.F.	Free parasites which had been stored frozen
I	Insoluble components of freeze-thawed free parasites
S	Soluble components of freeze-thawed free parasites
UnB	Unbroken free parasites
F-T-W	Unfractionated freeze-thawed free parasites
SC	Material washed from the surface of the unbroken free parasites

Table II

Relative mobilities of protein bands in a preparation washed from free parasites (left) or extracted from free parasites by freezing and thawing three times (right).

Wash Off Preparation	Freeze-thawed Parasites (Kreier et al.,1976
0	0
2.7	3.6
-	-
12.	12
16.	17
20.	22
26.	25
-	28
33.	34
39.	39
-	45
47.	48
52.	-
56. } one thick band*	56
60.	60
63.	63
-	71
78.	75
81.	80
86.	86
-	-
100	100

*See Figure 9

List of Figures

Figure 1

The relationship of antigen dose to immunological response in weanling (40-50 g.) and young adult (70-90 g.) rats. A) The course of parasitaemia following challenge in 40-50 gram rats given four injections of various numbers of small free plasmodium berghei parasites (F.P.) without adjuvant. B) The course of parasitaemia following challenge in 40-50 gram rats given four injections of various numbers of small free Plasmodium berghei parasites (F.P.) with saponin. C) The course of parasitaemia in 70-90 gram rats given four injections of various numbers of small free parasites (F.P.) without adjuvants. D) The course of parasitaemia in 70-90 gram rats given four injections of various numbers of small free parasites (F.P.) with saponin. The control rats received red blood cell membrane antigen (RBCM) in a value equal to the volume of parasites given to the immunized rats. All free parasites were freeze-thawed three times before injection.

Figure 2

The relationship between parasitaemia following challenge and time after immunization at which challenge takes place. The figure shows the course of parasitaemia after challenge in 70-90 gram rats given a single dose of 5 x 10^8 freeze-thawed free parasites (F.P.) with or without saponin and challenged at one week (A and E) 2 weeks (B and F) four weeks (C and G) or eight weeks (D and H). Each challenged group had its corresponding control group which received red blood cell membranes (RBCM).

Figure 3

Comparison of the immunogenicity of freshly prepared free parasite antigen (Fresh F.P.) and free parasite antigen which had been stored frozen (F.P. stored frozen) for two weeks. The control group received red blood cell membranes (RBCM). The test rats weighed 250-280 grams and no adjuvant was added to the parasite preparation. The free parasites were freeze-thawed three times before injection.

Figure 4

Effect of maturity of the rat upon immunization. The course of parasitaemia in 40-50 gram rats given four injections 5×10^8 free parasites (F.P.) or red blood cell membranes (RBCM) without (A) or with (D) saponin (A and D). The course of parasitaemia in 70-90 gram rats given four injections of 5×10^8 free parasites (F.P.) or red blood cell membranes (RBCM) without (B) or with (E) saponin (B and E). The course of parasitaemia in 250-280 gram rats given four injections of 5×10^8 free parasites (F.P.) or red blood cell membranes without (C) or with (F) saponin (C and F). All parasites were freeze-thawed three times before injection.

Figure 5

Comparison of the relative effects of saponin (A) and Freund's Complete Adjuvant (FCA) (B) on the immune response. These 70-90 gram rats received either four injections of 5×10^8 freeze-thawed free parasites (F.P.) in saponin or in FCA. The control animals received four injections of red blood cell membranes (RBCM) with the appropriate adjuvant.

Figure 6

Comparison of the relative immunogenicity of the soluble (Soluble F.P.) and the insoluble (Insoluble F.P.) components of freeze-thawed free parasites. The positive control rats received one injection of unfractionated material from 5×10^8 freeze-thawed parasites (F.P.) while the negative control rats received red blood cell membranes (RBCM).

Figure 7

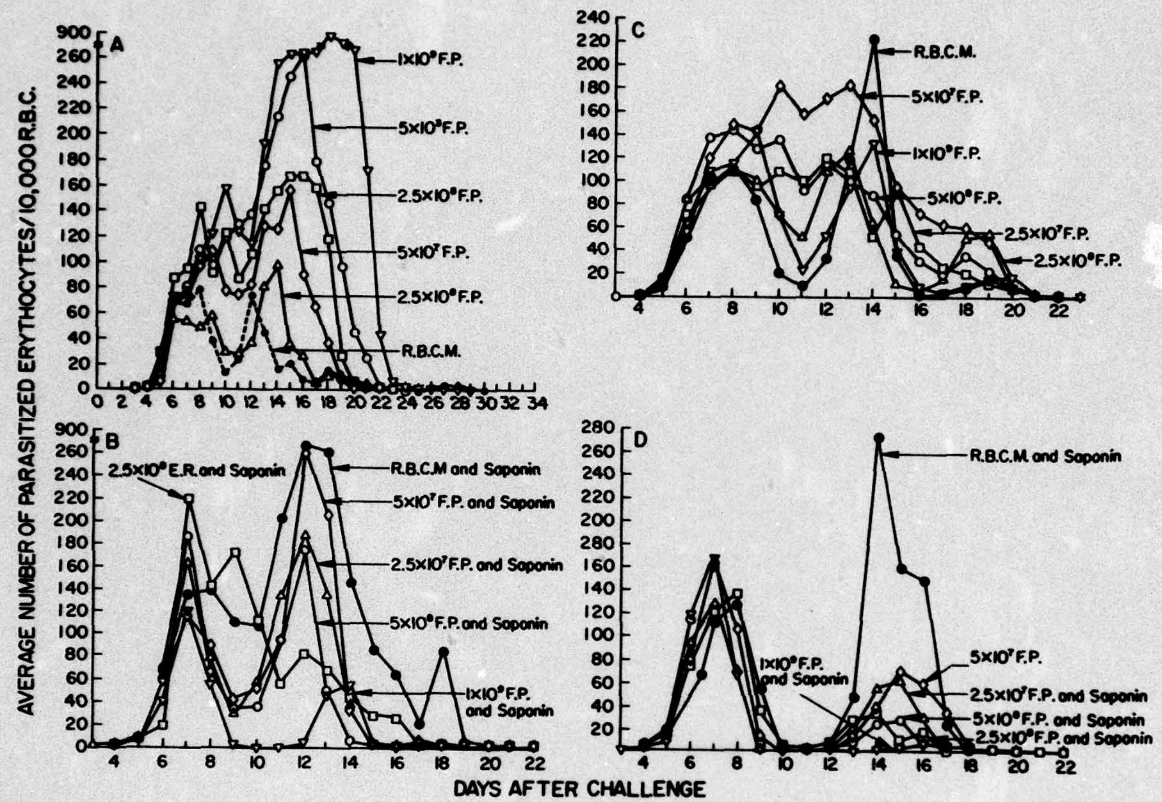
Comparison of the relative immunogenicity of whole unbroken (Unbroken F.P.) and freeze-thawed (Broken F.P.) free parasites. The antigens were injected with saponin (A) or with FCA (B). Control rats received red blood cell membranes (RBCM) in the appropriate adjuvants.

Figure 8

Disc gel electrophoresis patterns obtained with the soluble portions of freeze-thawed free parasites (left column) and of the soluble material washed from free parasites (right column).

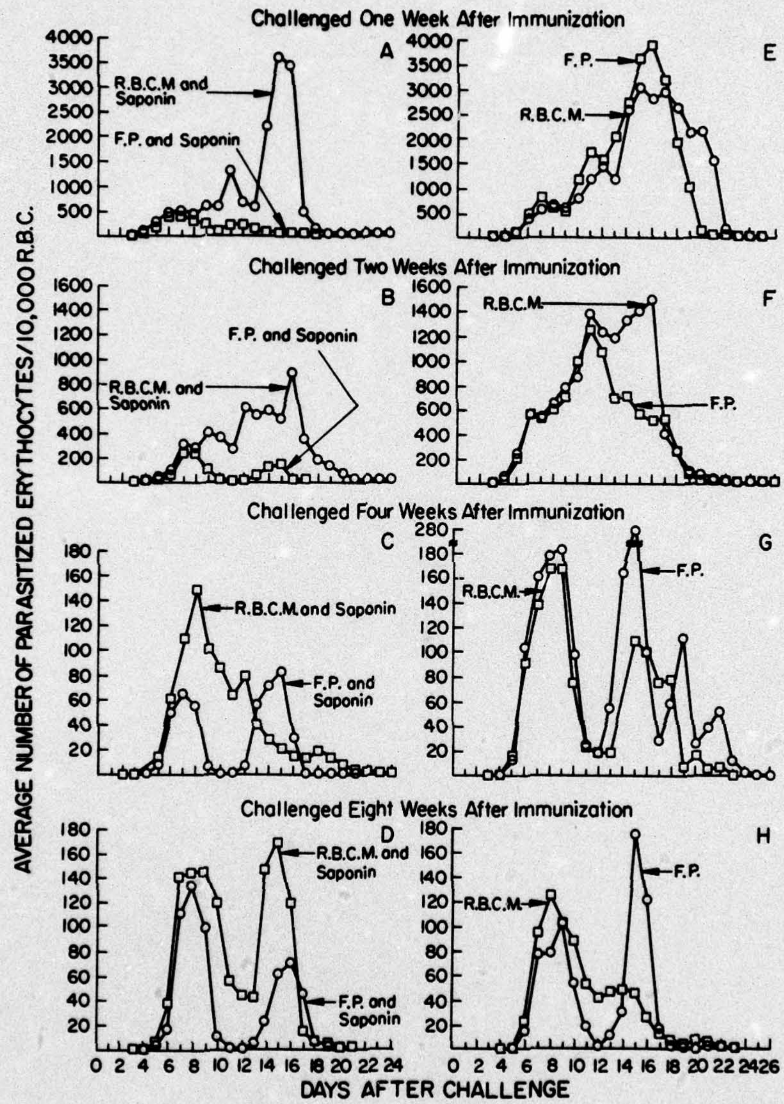
Figure 9

Comparison of the immunogenicity of an antigen washed from the surface of free parasites (wash off antigen) to that of the unfractionated parasite antigen (F.P.). The control rats received red blood cell membranes (RBCM).



A
1X10⁹ F.P.
5X10⁸ F.P.
2.5X10⁸ F.P.
5X10⁷ F.P.
2.5X10⁸ F.P.
R.B.C.M.
B
2.5X10⁸ E.R. and Saponin
R.B.C.M and Saponin
5X10⁷ F.P. and Saponin
2.5X10⁷ F.P. and Saponin
5X10⁸ F.P. and Saponin
1X10⁹ F.P. and Saponin
C
R.B.C.M.
5X10⁷ F.P.
1X10⁹ F.P.
5X10⁸ F.P.
2.5X10⁷ F.P.
2.5X10⁸ F.P.
D
R.B.C.M. and Saponin
1X10⁹ F.P. and Saponin
5X10⁷ F.P.
2.5X10⁷ F.P. and Saponin
5X10⁸ F.P. and Saponin
2.5X10⁸ F.P. and Saponin
AVERAGE NUMBER OF PARASITIZED ERYTHOCYTES/10,000 R.B.C.
DAYS AFTER CHALLENGE

Figure 1



Challenged One Week After Immunization
A
R.B.C.M and Saponin
F.P. and Saponin
E
F.P.
R.B.C.M.
Challenged Two Weeks After Immunization
B
F.P. and Saponin
R.B.C.M. and Saponin
F
R.B.C.M.
F.P.
Challenged Four Weeks After Immunization
C
R.B.C.M. and Saponin
F.P. and Saponin
G
R.B.C.M.
F.P.
Challenged Eight Weeks After Immunization
D
R.B.C.M. and Saponin
F.P. and Saponin
H
R.B.C.M.
F.P.
AVERAGE NUMBER OF PARASITIZED ERYTHROCYTES/10,000 R.B.C.
DAYS AFTER CHALLENGE

Figure 2

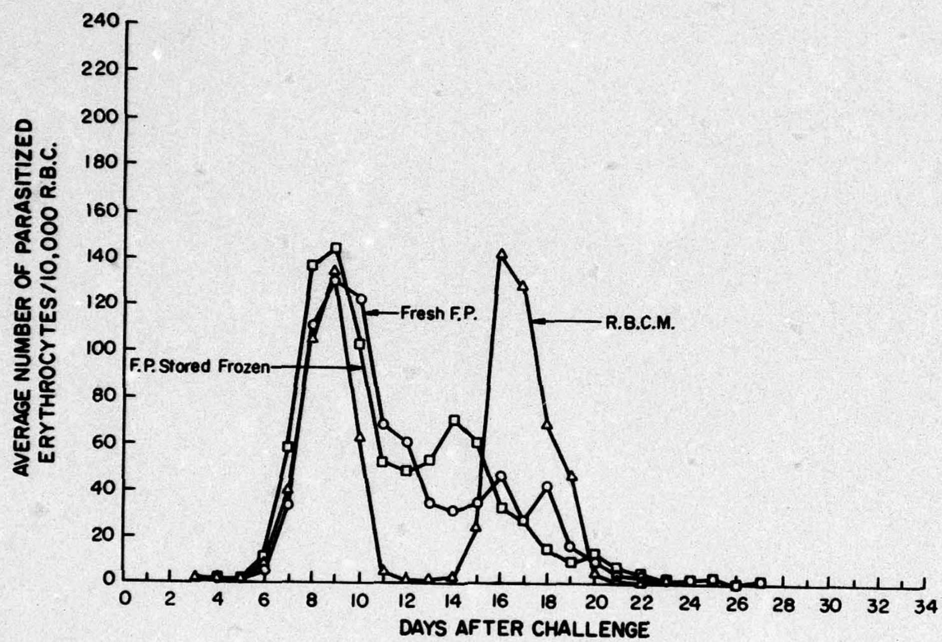

AVERAGE NUMBER OF PARASITIZED ERYTHROCYTES/10,000 R.B.C.
240
220
200
180
160
140
120
100
80
60
40
20
0
Fresh F.P.
R.B.C.M.
F.P. Stored Frozen
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
DAYS AFTER CHALLENGE

Figure 3

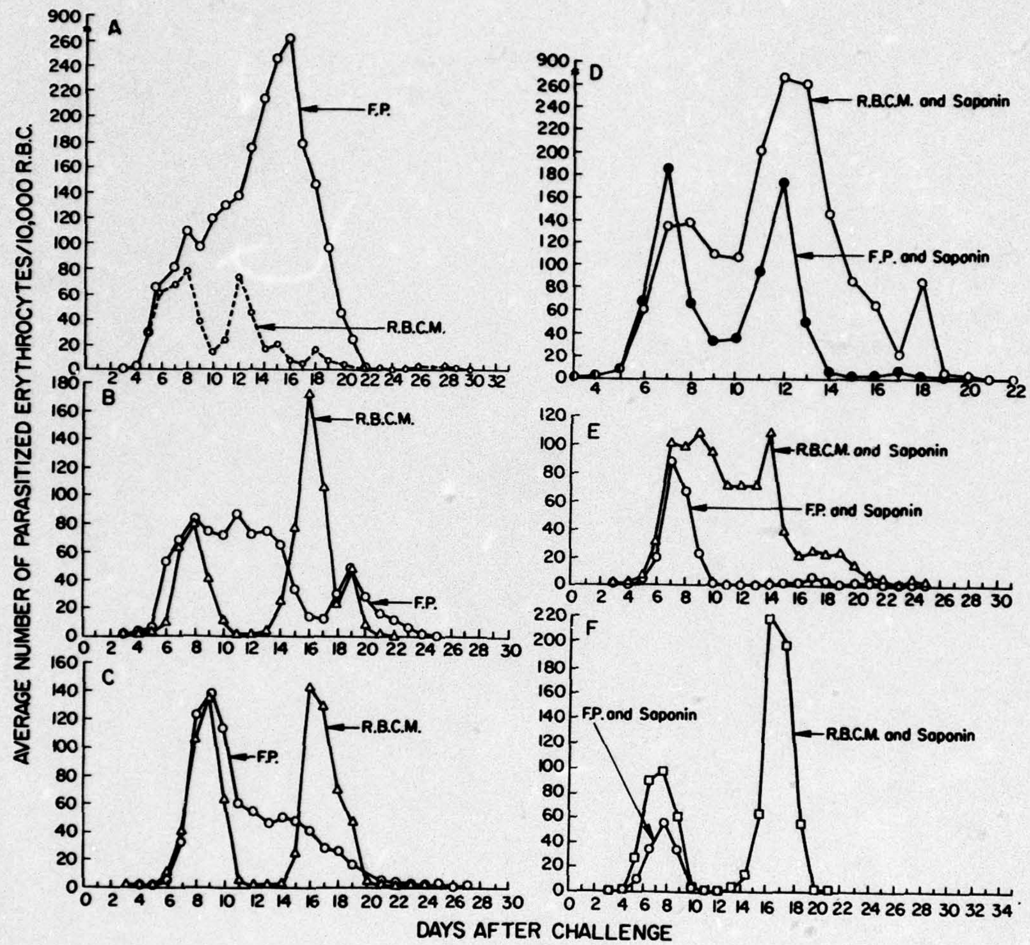

A
F.P.
R.B.C.M.
B
R.B.C.M.
F.P.
C
R.B.C.M.
F.P.
D
R.B.C.M. and Saponin
F.P. and Saponin
E
R.B.C.M. and Saponin
F.P. and Saponin
F
F.P. and Saponin
R.B.C.M. and Saponin
AVERAGE NUMBER OF PARASITIZED ERYTHROCYTES/10,000 R.B.C.
DAYS AFTER CHALLENGE

Figure 4

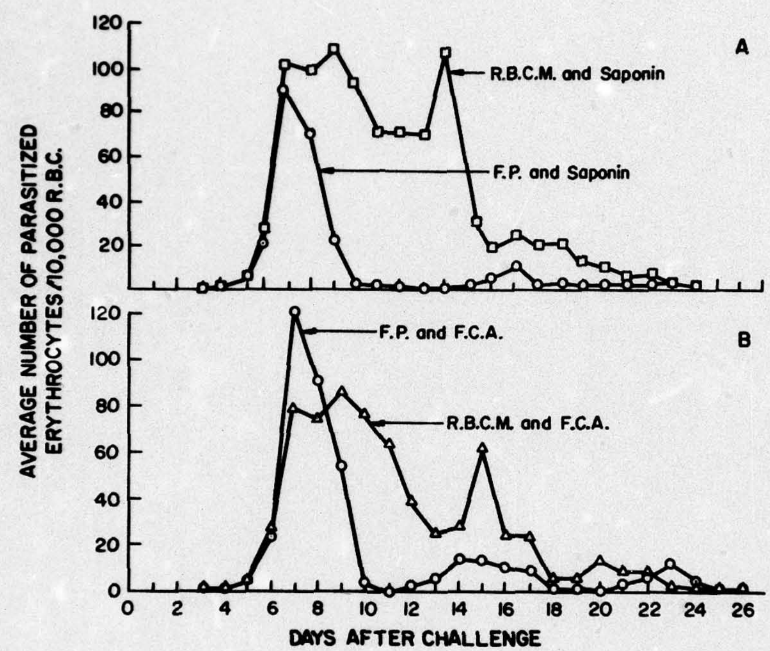

AVERAGE NUMBER OF PARASITIZED ERYTHROCYTES/10,000 R.B.C.
A
R.B.C.M. and Saponin
F.P. and Saponin
B
F.P. and F.C.A.
R.B.C.M. and F.C.A.
DAYS AFTER CHALLENGE

Figure 5

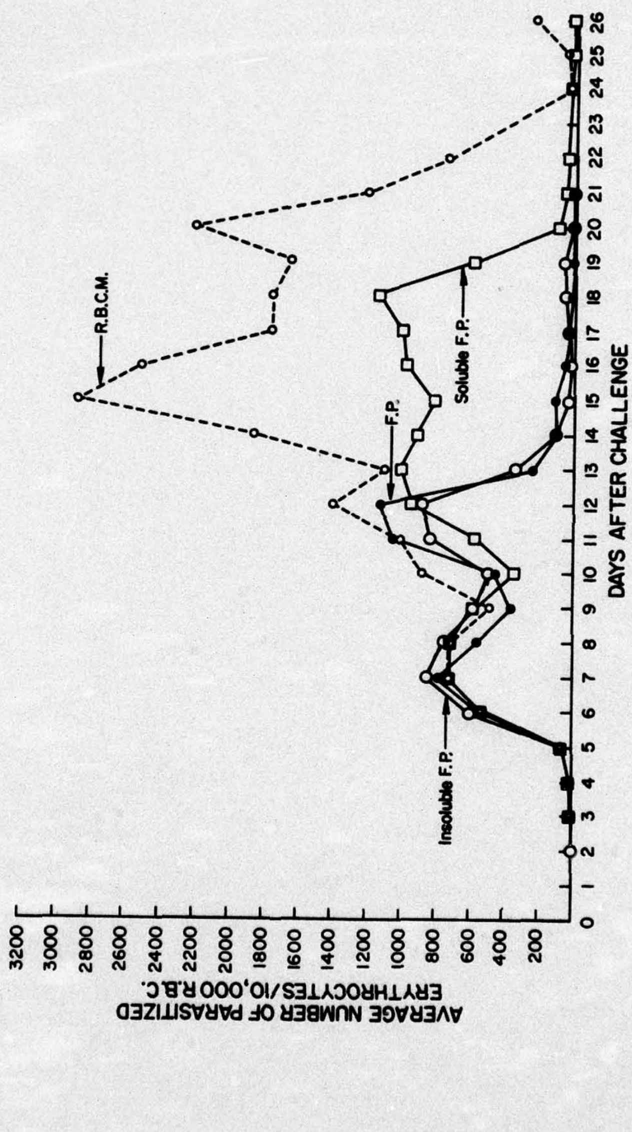

AVERAGE NUMBER OF PARASITIZED ERYTHROCYTES/10,000 R.B.C.
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
DAYS AFTER CHALLENGE
R.B.C.M.
F.P.
Soluble F.P.
Insoluble F.P.

Figure 6

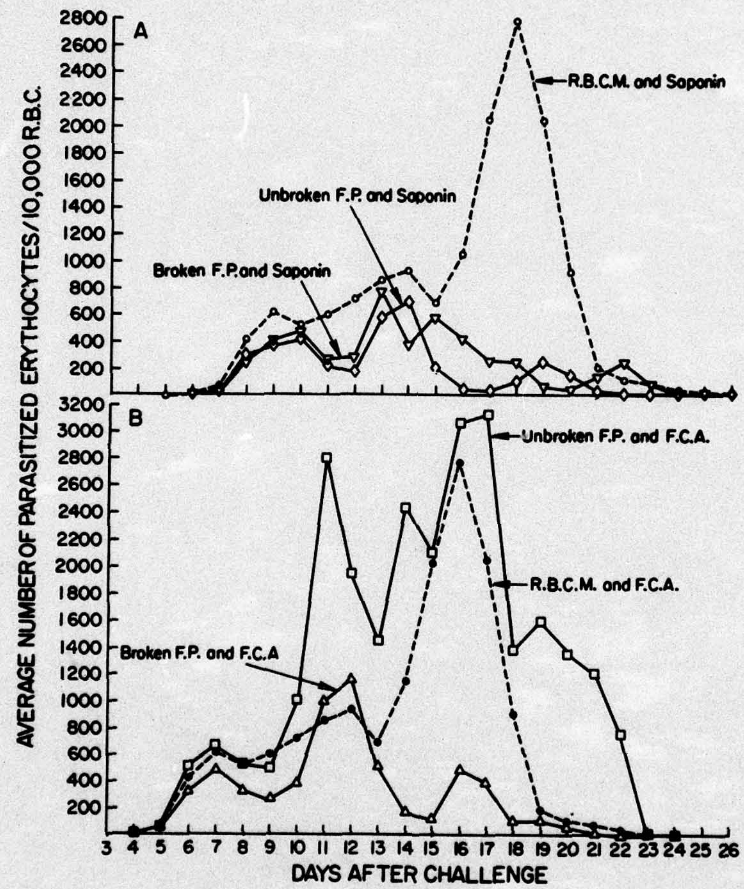

A
B
R.B.C.M. and Saponin
Unbroken F.P. and Saponin
Broken F.P. and Saponin
Unbroken F.P. and F.C.A.
R.B.C.M. and F.C.A.
Broken F.P. and F.C.A
AVERAGE NUMBER OF PARASITIZED ERYTHROCYTES/10,000 R.B.C.
DAYS AFTER CHALLENGE

Figure 7

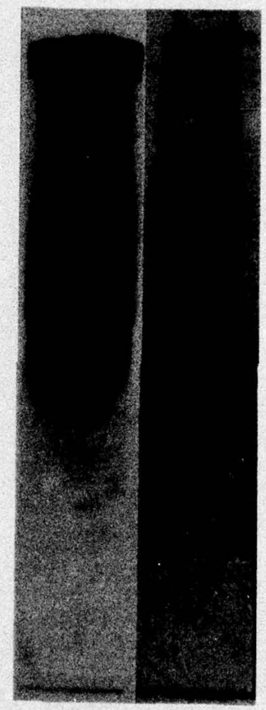


Figure 8

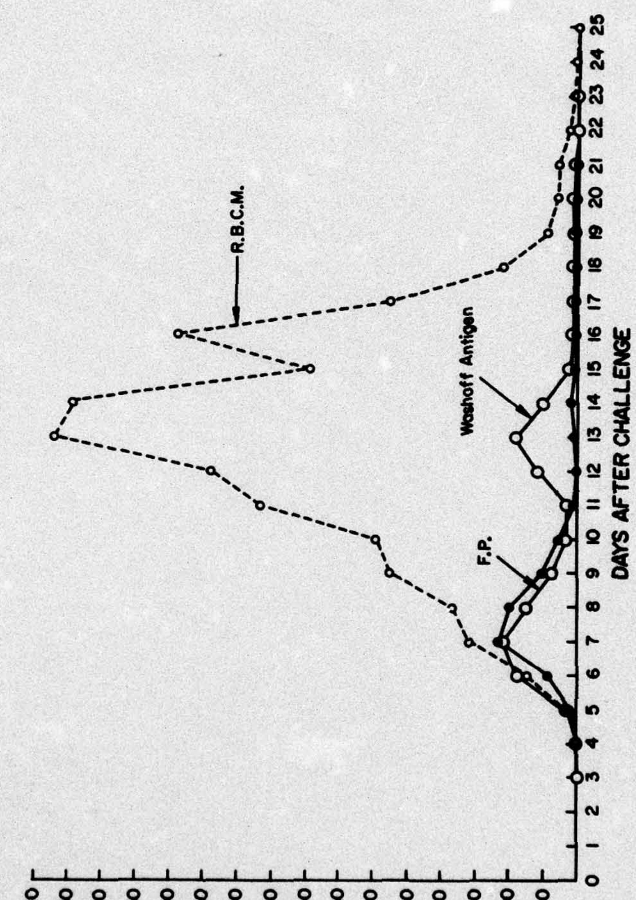

R.B.C.M.
Washoff Antigen
F.P.
DAYS AFTER CHALLENGE
AVERAGE NUMBER OF PARASITIZED ERYTHROCYTES/10,000 R.B.C.

Figure 9

AD-A035 042   ISOLATION AND CHARACTERIZATION OF PLASMODIAL AND BABESIAL ANTIGENS(U) OHIO STATE UNIV RESEARCH FOUNDATION COLUMBUS   J P KREIER 15 JAN 77   2/2

UNCLASSIFIED   OSURF-3365-3-REV-77 DADA-17-72-C-2072   F/G 6/13   NL

SUPPLEMENTARY

INFORMATION

END
DATE
FILMED
11-84
DTIC

MICROCOPY RESOLUTION TEST CHART

NATIONAL BUREAU OF STANDARDS-1963-A

SUPPLEMENTARY

INFORMATION

Errata

AD-A035 042

Pages 22 and 32 are blanks.

DTIC-DDAC
30 Aug 84